



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> C07H 19/00, 21/00, 21/04, C12N 1/21, C12Q 1/02, 1/10, 1/18, 1/24, G01N 33/569	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/18225</b> <b>(43) International Publication Date:</b> 22 May 1997 (22.05.97)
<b>(21) International Application Number:</b> PCT/US96/18504 <b>(22) International Filing Date:</b> 14 November 1996 (14.11.96)  <b>(30) Priority Data:</b> 60/006,733 14 November 1995 (14.11.95) US  <b>(71) Applicant (for all designated States except US):</b> THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> MILLER, Samuel, I. [US/US]; Health Sciences Building, K140, Box 357710, University of Washington, 1959 Pacific Street, N.E., Seattle, WA 98195 (US).  <b>(74) Agent:</b> MEIKLEJOHN, Anita, L.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).	<b>(81) Designated States:</b> AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> SALMONELLA SECRETED PROTEINS AND USES THEREOF  <b>(57) Abstract</b>  Substantially pure <i>Salmonella</i> secreted proteins (Ssp), the secretion of which is dependent upon the expression of PrgH; methods of diagnosing <i>Salmonella</i> infection; and live attenuated vaccine strains in which Ssp secretion is decreased.		

ATTORNEY DOCKET NUMBER: 8002-059-999  
SERIAL NUMBER: 09/645,415  
REFERENCE: EO

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

- 1 -

SALMONELLA SECRETED PROTEINS AND USES THEREOFStatement as to Federally Sponsored Research

This invention was made with Government support  
5 under AI34504 and AI30479 awarded by the National  
Institutes of Health. The Government has certain rights  
in the invention.

Background of the Invention

The invention relates to virulence factors of  
10 *Salmonella typhimurium*.

*Salmonella typhimurium* (*S. typhimurium*) enter  
epithelial cells by a process termed bacterial-mediated  
endocytosis. *S. typhimurium* stimulates these normally  
nonphagocytic cells to undergo significant cytoskeletal  
15 rearrangements that are visualized as localized membrane  
ruffling adjacent to the bacteria. Bacteria are then  
internalized via membrane-bound vacuoles formed from the  
membrane ruffles.

Several *S. typhimurium* loci have been identified  
20 that are required for the induction of bacterial-mediated  
endocytosis (BME) by epithelial cells. Many of these  
epithelial-cell signaling loci have a similar chromosomal  
location, clustered within a 40 kb "virulence island"  
located between 59 and 60 minutes on the *S. typhimurium*  
25 chromosome (Mills et al., *Mol. Microbiol.* 15:749-759,  
1995). *InvJ* is a *S. typhimurium* gene which is thought  
to encode a secreted protein necessary for BME (Collazo  
et al., *Mol. Microbiol.* 15:25-38, 1995).

Summary of the Invention

30 The invention features proteins involved in  
*Salmonella typhimurium* virulence and/or  
bacterial-mediated endocytosis. The genes encoding these  
proteins have now been cloned and their corresponding  
gene products characterized. Accordingly, the invention

- 2 -

features a substantially pure DNA encoding a *Salmonella* secreted protein (Ssp). By the term "Salmonella secreted protein" is meant a *Salmonella*-derived protein, the secretion of which is dependent on the expression of

5 PrgH. In preferred embodiments the invention features substantially pure DNA encoding a *Salmonella typhimurium* secreted protein. By *Salmonella typhimurium* secreted protein is meant as *Salmonella typhimurium* derived protein, the secretion of which is dependent on the

10 expression of PrgH.

One aspect of the invention features a substantially pure DNA molecule which includes the SspB gene; preferably, the DNA includes the DNA sequence of SEQ ID NO: 1, or degenerate variants thereof encoding the

15 amino acid sequence of SEQ ID NO: 5. In another aspect the invention features a substantially pure DNA molecule which includes the SspC gene; preferably, the DNA includes the DNA sequence of SEQ ID NO: 2, or degenerate variants thereof encoding the amino acid sequence of SEQ

20 ID NO: 6. In another aspect the invention features a substantially pure DNA molecule which includes the SspD gene; preferably, the DNA includes the DNA sequence of SEQ ID NO: 3, or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 7. In another aspect

25 the invention features a substantially pure DNA molecule which included the SspA gene; preferably, the DNA includes the DNA sequence of SEQ ID NO: 4, or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 8. The invention also features a substantially

30 pure DNA molecule which includes the SspB, SspC, SspD, and SspA genes; preferably, the DNA includes the DNA sequence of SEQ ID NO: 15. The invention also features a substantially pure DNA molecule which includes the SspH gene; preferably, the DNA includes the DNA sequence of

35 SEQ ID NO: 13, or degenerate variants thereof encoding

- 3 -

the amino acid sequence of SEQ ID NO: 14. The invention also features a substantially pure DNA molecule which includes the *Salmonella* tyrosine phosphatase A (stpA) gene; preferably, the DNA includes the DNA sequence of  
5 SEQ ID NO: 10, or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO:12.

The invention also features a cell into which has been introduced substantially pure DNA encoding an Ssp (or a mutant variant thereof). The substantially pure  
10 DNA can be introduced as a portion of a plasmid or other autonomously replicating molecule. In addition the substantially pure DNA can be introduced by homologous recombination. Preferably, the bacterial cell is a *Salmonella* cell; more preferably the bacterial cell is a  
15 *Salmonella typhimurium* cell. Cells into which have been introduced substantially pure DNA encoding an Ssp (or mutant variant thereof) can be used as a source of purified Ssp.

The invention includes a substantially pure SspC  
20 polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 6 or an active fragment thereof and a substantially pure SspD polypeptide, e.g., a polypeptide which includes an amino acid sequence  
25 substantially identical to the amino acid sequence of SEQ ID NO: 7 or an active fragment thereof. The invention includes a substantially pure SspB polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ  
30 ID NO: 5 (incomplete protein sequence) or an active fragment thereof and a substantially pure SspA polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 8 (incomplete protein sequence) or  
35 an active fragment thereof. The invention includes a

- 4 -

substantially pure full-length SspB polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 5 (incomplete protein sequence) and the remainder of the SspB sequence. Full-length SspA and SspB genes can be isolated by those skilled in the art using the partial DNA sequences disclosed herein. The invention also includes a substantially pure full-length SspA polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 8 (incomplete protein sequence) and the remainder of the SspA sequence. The invention also features An active fragment of an Ssp B polypeptide or an SspC polypeptide or an SspD polypeptide is defined as an SspB, SspC, or an SspD polypeptide, respectively, at least 50 amino acids, preferably at least 25 amino acids, more preferably at least 10 amino acids in length having the ability to induce BME in the absence of the full-length version of the corresponding protein. In other preferred embodiments the SspB, SspC, SspD or SspA polypeptide is able to translocate into an epithelial cell, preferably a human epithelial cell. Translocation can be assayed using any suitable assay, e.g., the assay of Sogy et al. (*Molecular Microbiol.* 14:583:94, 1994).

25       The invention also includes a substantially pure SspH polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO:14, or a biologically active fragment thereof.

30       The invention also includes a substantially pure IagB polypeptide, e.g., a polypeptide which includes an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO:11, or a biologically active fragment thereof.

- 5 -

Also within the invention is an antibody which binds to a Ssp, e.g., a polyclonal or monoclonal antibody which specifically binds to an epitope of Ssp. Polyclonal and monoclonal antibodies produced against the

5 polypeptides of the invention can be used as diagnostic or therapeutic agents. The invention encompasses not only an intact monoclonal antibody, but also an immunologically-active antibody fragment, e.g., a Fab or (Fab)<sub>2</sub> fragment; an engineered single chain Fv molecule.

10 In preferred embodiments, the antibody may be linked to a detectable label, e.g. a radioactive label, fluorescent label, paramagnetic label, or colorimetric label.

The invention also includes a method of detecting a *Salmonella* infection in a mammal which includes the

15 steps of contacting a biological sample derived from the mammal, e.g., a human patient, with a Ssp-specific antibody and detecting the binding of the antibody to a Ssp in the sample. Antibody binding indicates that the mammal is infected with *Salmonella*. The presence of

20 *Salmonella* in a biological sample may also be detected using a method which includes the steps of contacting the sample with a Ssp-encoding DNA, or the complement thereof, under high stringency conditions and detecting the hybridization of the DNA to nucleic acid in the

25 sample. Hybridization indicates the presence of *Salmonella* in the biological sample. By "high stringency" is meant DNA hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65°C at a salt

30 concentration of approximately 0.1 × SSC. For example, high stringency conditions may include hybridization at about 42°C in the presence of about 50% formamide; a first wash at about 65°C with about 2 × SSC containing 1% SDS; followed by a second wash at about 65°C with about

35 0.1 × SSC.

- 6 -

The invention also features a method for detecting the presence of antibodies to an Ssp using all or part of an Ssp protein. The method includes contacting a biological sample with the Ssp protein and measuring the binding of the Ssp protein to an antibody present in the sample.

The invention also features a method of targeting an antigen to an epithelial cell in a mammal which includes the steps of linking the antigen to an Ssp, e.g., SspC or SspD, or active fragment thereof, to produce a Ssp chimeric antigen and administering the chimeric antigen to the mammal.

A method of inducing a cytotoxic T cell immune response in a mammal is also within the invention. This method includes the steps of linking the antigen to an Ssp or active fragment thereof to produce a Ssp chimeric antigen and contacting an antigen-presenting cell, e.g., a Class I major histocompatibility complex (MHC) antigen-expressing cell, with the chimeric antigen.

The invention also features a vaccine which includes a bacterial cell, the virulence of which is attenuated by decreased secretion of a Ssp, and a method of vaccinating an mammal, e.g., a human patient, against a *Salmonella* infection by administering such a vaccine. Preferably, the bacterial cell is a *Salmonella typhimurium* cell, e.g., a *Salmonella enteriditis* cell, or a *Salmonella typhi* cell. A live *Salmonella* cell in which a gene encoding a heterologous antigen is inserted into a Ssp-encoding gene is also included in the invention.

Also within the invention is a substantially pure StpA polypeptide and a method of dephosphorylating a protein which includes the steps of contacting the protein, e.g., a protein at least one tyrosine of which is phosphorylated, with a StpA polypeptide or an active fragment thereof. An active fragment of StpA is defined



- 7 -

as a *Salmonella*-derived polypeptide at least 10 amino acids in length which is capable of removing a phosphate group from a tyrosine residue.

The invention features live *Salmonella*

- 5 (particularly *Salmonella typhimurium*) vaccines in which one or more gene required for BME is mutated so as reduce their activity. Among the genes which can be mutated are *SspB*, *SspC*, and *SspD*. Although *SspA* appears not to be required for BME, it may be useful to mutate this gene as  
10 well (preferably in combination with mutation of one or more of the other *Ssp* genes). Any mutation of these genes which decreases function, including complete or partial deletion and one or more point mutations may be useful. In addition, function of *Ssp* gene may be  
15 impaired by altering its control region.

The invention provides a *Salmonella* vaccine which does not cause transient bacteremia. In general, the invention features a bacterial cell, preferably a *Salmonella* cell, e.g., a *S. typhi*, *S. enteritidis*  
20 *typhimurium*, or *S. cholerae-suis* cell, the virulence of which is attenuated by a first mutation in an *Ssp* gene. The preferred mutations are loss of function mutations. However, functions causing partial loss of function may be useful if virulence is adequately reduced. Such a  
25 bacterial cell can be used as a vaccine to immunize a mammal against salmonellosis.

The *Salmonella* cell may be of any serotype, e.g., *S. typhimurium*, *S. paratyphi A*, *S. paratyphi B*, *S. paratyphi C*, *S. pylorum*, *S. dublin*, *S. heidelberg*, *S. newport*, *S. minnesota*, *S. infantis*, *S. virchow*, or *S. panama*.

The first mutation may be a non-revertible null mutation in one or more of the following genes: *SspB*, *SspC*, or *SspD*. Preferably, the mutation is a deletion  
35 of at least 100 nucleotides; more preferably, the

- 8 -

mutation is a deletion of at least 500 nucleotides; even more preferably, the mutation is a deletion of at least 750 nucleotides. Mutations in the *prgH* gene or the *prgH* operon can be used for the same purpose.

5 In preferred embodiments loss or function (partial or complete) is due to decreased expression as a result of a change or mutation, e.g., a deletion, (preferably a non-revertible mutation) at the promoter or other regulatory element of *SspB*, *SspC*, or *SspD* (or some  
10 combination thereof).

In another aspect, the invention features a vaccine including a bacterial cell which is attenuated by decrease of expression of a *Ssp* virulence gene.

The invention also features a live *Salmonella*  
15 cell, or a substantially purified preparation thereof, e.g., a *S. typhi*, *S. enteritidis* typhimurium, or *S. cholerae-suis* cell, in which there is inserted into a virulence gene, e.g., an *Ssp* gene, a gene encoding a heterologous protein, or a regulatory element thereof.

20 In another aspect the invention includes a method of vaccinating an animal, e.g., a mammal, e.g., a human, against a disease caused by a bacterium, e.g., *Salmonella*, including administering a vaccine of the invention.

25 By "vaccine" is meant a preparation including materials that evoke a desired biological response, e.g., an immune response, in combination with a suitable carrier. The vaccine may include live organism, in which case it is usually administered orally, or killed  
30 organisms or components thereof, in which case it is usually administered parenterally. The cells used for the vaccine of the invention are preferably alive and thus capable of colonizing the intestines of the inoculated animal.

- 9 -

By "mutation" is meant any change (in comparison with the appropriate parental strain) in the DNA sequence of an organism. These changes can arise e.g., spontaneously, by chemical, energy e.g., X-ray, or other forms of mutagenesis, by genetic engineering, or as a result of mating or other forms of exchange of genetic information. Mutations include e.g., base changes, deletions, insertions, inversions, translocations or duplications.

10 A mutation attenuates virulence if, as a result of the mutation, the level of virulence of the mutant cell is decreased in comparison with the level in a cell of the parental strain, as measured by (a) a significant (e.g., at least 50%) decrease in virulence in the mutant strain compared to the parental strain, or (b) a significant (e.g., at least 50%) decrease in the amount of the polypeptide identified as the virulence factor in the mutant strain compared to the parental strain.

A non-revertible mutation, as used herein, is a mutation which cannot revert by a single base pair change, e.g., deletion or insertion mutations and mutations that include more than one lesion, e.g., a mutation composed of two separate point mutations.

Heterologous protein, as used herein, is a protein that in wild type, is not expressed or is expressed from a different chromosomal site, e.g., a heterologous protein is one encoded by a gene that has been inserted into a second gene.

A substantially purified preparation of a bacterial cell is a preparation of cells wherein contaminating cells without the desired mutant genotype constitute less than 10%, preferably less than 1%, and more preferably less than 0.1% of the total number of cells in the preparation.

- 10 -

A substantially pure DNA, as used herein, refers to a nucleic acid sequence, segment, or fragment, which has been purified from the sequences which flank it in a naturally occurring state, e.g., a DNA which has been  
5 removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in the genome in which it naturally occurs. The term also applies to DNA which has been substantially purified from other components which naturally accompany  
10 the DNA, e.g., DNA which has been purified from proteins which naturally accompany it in a cell.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments and from the claims.

15 By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%,  
20 preferably 85%, more preferably 90%, and most preferably 95% sequence identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 10 amino acids, preferably at least 20 amino acids, more  
25 preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably  
30 100 nucleotides.

Sequence identity is typically measured using sequence analysis software (e.g., Sequence analysis software package of the genetics computer group, university of Wisconsin biotechnology center, 1710  
35 university avenue, Madison, WI 53705). Such software

- 11 -

matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially pure polypeptide" is meant a Ssp polypeptide which has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60% Ssp by weight. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, Ssp polypeptide. A substantially pure Ssp polypeptide may be obtained, for example, by extraction from a natural source (e.g., *Salmonella* bacterium); by expression of a recombinant nucleic acid encoding a Ssp polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., using column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components.

Accordingly, substantially pure polypeptides include those derived from one type of prokaryotic organism, e.g., *S. typhimurium*, but synthesized in *E. coli* or another prokaryotic organism.

By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring

- 12 -

genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously  
5 replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a  
10 recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence, e.g., a hybrid gene encoding a chimeric antigen.

Other features and advantages of the invention will be apparent from the following description of the  
15 preferred embodiments thereof, and from the claims.

#### Detailed Description

Fig. 1 is a diagram of the a genetic map of the 59-60 min region of the *S. typhimurium* chromosome and partial physical map of the restriction endonuclease  
20 sites of the *prgH* chromosomal region within the *hil* locus and related plasmids. The horizontal arrows indicate the direction of transcription of the *orf1*, *prgHIJK*, and *org* genes and of the neomycin promoter of the Tn5B50 insertions within the *hil* locus. The vertical arrows  
25 indicate and the location of the predicted start of transcription of the *prgHIJK* operon (small arrow) and the location of the two Tn5B50 insertions that define the *hil* locus (large arrows). The open triangle indicates the location of the *prgH1::TnphoA* insertion. Restriction  
30 endonuclease sites are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sac*I; Ss, *Ssp*I; V, *Eco*RV; X, *Xho*I.

Fig. 2 is a photograph of a Northern blot assay in which the *prgHIJK* and *org* transcripts are identified. Blot hybridization of a *prgH* (A), *prgI-J* (B) *prgK* (C),  
35 *org* (D), and *pagC* (E) DNA probe to RNA purified from

- 13 -

wild-type (Wt) and *phoP* constitutive ( $P^C$ ) *S. typhimurium* strains were grown aerobically to 0.5 optical density units. The bars indicate the RNA markers and are 9488, 6255, 3911, 2800, 1898, and 872 nucleotides (NT) in size from top to bottom.

Fig. 3 is a photograph of a primer extension analysis of RNA isolated from wild-type and  $PhoP^C$  *S. typhimurium* strains by using an oligonucleotide primer IB08 corresponding to nucleotides 1217 to 1199 of the *prgH* sequence. Lanes labeled "AGCT" represent dideoxy DNA-sequencing reactions. The lane labeled "wt" represents the products of a primer extension reaction initiated with primer IB08 and wild-type RNA as a template, and the lane labeled " $P^C$ " represents the products of a primer extension reaction initiated with the same primer and  $PhoP^C$  RNA as a template. Reverse transcription of wild-type RNA with primer IB08 resulted in an approximately 270-nucleotide product corresponding to a predicted transcriptional start at nucleotide 949 of the *prgH* sequence. Abbreviations: wt, wild type strain 14028s;  $P^C$ ,  $PhoP^C$  strain CS022.

Fig. 4A is a diagram showing the similarity and alignment of *prgI*, *mxhH*, and *yscF* predicted gene products.

Fig. 4B is a diagram showing the similarity and alignment of *prgJ* and *mxhI* predicted gene products.

Fig. 4C is a diagram showing the similarity and alignment of *prgK*, *mxhJ*, and *yscJ* predicted gene products. For Figs. 4A-4C, residues conserved among each of the predicted gene products are indicated with a plus (+); residues conserved among the *prgI* and either the *mxhH* or *yscF* predicted gene products and between the *prgK* and either the *mxhJ* or *yscJ* predicted gene products are indicated with an asterisk (\*). The location of the lipoprotein processing sites (Leu-Xaa-Gly-Cys) of the

- 14 -

*prgK*, *mxiJ*, and *yscJ* predicted gene products are indicated by underlining. Predicted protein sequences were compared using the GCG BLAST network service and ALIGN program (Feng et al., *J. Mol. Evol.* 35:351-360, 5 1987; Higgins et al., *CABIOS* 5:151-153, 1989).

Fig. 5 is a photograph of a SDS-PAGE gel.

*Salmonella* proteins found in the culture supernatant of stationary-phase *S. typhimurium* 14028s were compared to proteins isolated from lysed whole cells or cellular 10 fractions (membranes or intracellular soluble proteins). TCA precipitable material from 2 ml of supernatant from cultures of  $OD_{600} = 2.2$  was used. The whole cell, membrane, and soluble lanes contained material from 0.10 ml, 0.35 ml, and 0.15 ml of cells, respectively. 15 Proteins were fractionated in a 12% polyacrylamide gel by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The molecular masses of protein standards are indicated on the side of the gel as kDa.

Fig. 6 is a photograph of a SDS-PAGE gel showing a 20 comparison of culture supernatant proteins from *S. typhimurium* 14028s and culture supernatants from mutants which are defective in eucaryotic signaling. TCA precipitable material from 2 ml of bacterial culture supernatant was isolated at different times following 25 inoculation: mid-log,  $OD_{600} = 0.6$ ; late-log / early-stationary,  $OD_{600} = 1.1$ ; stationary,  $OD_{600} = 2.2$ . Proteins were fractionated in a 12% polyacrylamide gel by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The molecular masses of protein standards are indicated 30 on the side of the gel as kDa. wt, wild type (14028s);  $p^c$ ,  $PhoP^c$  (CS022);  $P^-$ ,  $PhoP^-$  (CS015);  $\Delta hil$  (CS451), deleted for the *hil* locus.

Fig. 7 is a photograph of a SDS-PAGE gel showing an analysis of *prgH::Tnp<sub>phoA</sub>* and complementation of the 35 insertion mutation by *pWKSH5*. TCA precipitable material



- 15 -

from 2 ml of supernatant from stationary phase cultures was fractionated in a 10% polyacrylamide gel by SDS-PAGE. Protein was stained with Coomassie Brilliant Blue R-250. The molecular masses of protein standards are indicated on the side of the gel as kDa. wt, wild-type (14028s); IB040, *prgH1::TnpHoA*; IB043, *prgH1::TnpHoA* with plasmid pWKSH5 containing a 5.1 kb insert of *S. typhimurium* DNA including *prgHIJK*. Supernatant protein bands complemented by pWKSH5 are indicated by arrows (87 kDa and 65 kDa) and a bracket (three bands in the 35-40 kDa range).

Fig. 8 is a photograph of a SDS-PAGE gel showing *Salmonella* secreted proteins (Ssp) concentrated from supernatants of different strains. Each lane contains Ssp collected from 2 ml of culture supernatant. Lanes 1: wild-type *S. typhimurium* SL1344; 2: EE638 (*lacZY11-6*); 3: EE633 (*lacZY4*); 4: VB122 (*hila::kan-112*); 5: EE637 (*invF::lacZY11-5*); 6: IB040 (*prgH1::TnpHoA*) St: molecular weight standard. Sizes of protein bands are given in kDa. \* marks a protein band which was variably present in different preparations of Ssp from the same strains.

Fig. 9 is a diagram showing the chromosomal organization of the *sspBCDA* genes and phenotypes of mutants *sspC::lacZY4* (EE633) and *sspA::lacZY11-6* (EE638). The chromosomal location of *ssp* with respect to *spaT* and *prgH* is shown. An asterisk (\*) indicates partially sequenced genes. Restriction sites in parentheses have only been mapped in the left region of the 11 kb *EcoRI* fragment. Abbreviations of restriction sites are: E: *EcoRI*, B: *BamHI*, P: *PvuII*, N: *NcoI*. Invasion of epithelial cells by different *S. typhimurium* strains is given as the percentage of the bacterial inoculum surviving gentamicin treatment. Values represent means and standard errors of the means of three independent experiments, each performed in triplicate. Presence or

- 16 -

absence of *Salmonella* secreted proteins SspA, SspC and SspD in culture supernatants of different strains is indicated by + or -, respectively. The molecular weights in kDa of these Ssp are shown in parentheses.

5           Fig. 10 is a diagram showing a complementation analysis of EE638. Complementing fragments of chromosomal DNA in a low-copy plasmid are shown according to the chromosomal map. Designations of the plasmids are given in brackets on the left. The positions of the *lac* promoter (*P<sub>lac</sub>*) are indicated.  $\Delta$  indicates a deletion.

10           Fig. 11 is a photograph of an immunoblot analysis of various strains for expression and secretion of Ssp87. Total cellular proteins from bacteria collected from 0.2 ml of cultures were loaded in lanes designated "C",  
15           supernatant proteins from 0.2 ml bacterial culture supernatants were loaded in lanes designated "S". 1: wild type *S. typhimurium*; 2: CS022 (*PhoP<sup>C</sup>*); 3: IB040 (*prgH1::TnphoA*); 4: CS451 ( $\Delta$ *h1::Tn5-428*); 5: EE638 (*sspC::lacZY11-6*); 6: EE633 (*sspA::lacZY4*).

20           Fig. 12 is a diagram showing a comparison of the deduced partial amino acid sequence of SspB with the *S. flexneri* homologue IpaB. Bars indicate identical residues, dots indicate gaps introduced in order to maximize similarity according to the GAP program of the  
25           GCG package.

            Fig. 13 is a diagram showing a comparison of the deduced amino acid sequences of SspC with the *S. flexneri* homologues IpaC. Bars indicate identical residues, dots indicate gaps introduced in order to maximize similarity  
30           according to the GAP program of the GCG package.

            Fig. 14 is a diagram showing a comparison of the deduced amino acid sequences of SspD with the *S. flexneri* homologues IpaD. Bars indicate identical residues, dots indicate gaps introduced in order to maximize similarity  
35           according to the GAP program of the GCG package.

- 17 -

Fig. 15 is a diagram of the amino-terminal sequence derived from the 5'-region of *sspA*. Amino acids determined by amino-terminal sequencing of *SspC* and *SspA* are underlined.

5            Fig. 16 is a photograph of a SDS-PAGE gel showing total soluble *Ssp* collected from 2 ml of culture supernatants of wild type *S. typhimurium* SL1344 and EE638 (*sspC*::*lacZY11-6*) transformed with various plasmids. Lanes 1: SL1344 [pWSK29]; 2: EE638 [pWSK29]; 3: EE638  
10 [pCH004 (*sspC*)]; 4: EE638 [pCH005 (*sspCD*)]; 5: EE638 [pCH006 (*sspD*)]; 6: EE638 [pCH002 (*sspCDA*)]; 7: SL1344 [pCH002 (*sspCDA*)]. Lanes 8 and 9 contain soluble *Ssp* from SL1344 [pWSK29] and EE638 [pWSK29], respectively. The sizes of the protein bands are given in kDa. An  
15 asterisk (\*) indicates a protein band which was variably present in different preparations of *Ssp* from the same strains.

Fig. 17 is a photograph of an SDS-PAGE gel showing insoluble *Ssp* precipitates collected from 2 ml of culture  
20 supernatants of wild type *S. typhimurium* SL1344 and EE638 (*sspC*::*lacZY11-6*) transformed with various plasmids. Lanes 1: SL1344 [pWSK29]; 2: EE638 [pWSK29]; 3: EE638 [pCH004 (*sspC*)]; 4: EE638 [pCH005 (*sspCD*)]; 5: EE638 [pCH006 (*sspD*)]; 6: EE638 [pCH002 (*sspCDA*)]; 7: SL1344  
25 [pCH002 (*sspCDA*)]. Lanes 8 and 9 contain soluble *Ssp* from SL1344 [pWSK29] and EE638 [pWSK29], respectively. The sizes of the protein bands are given in kDa. An asterisk (\*) indicates a protein band which was variably present in different preparations of *Ssp* from the same  
30 strains.

Fig. 18 is a diagram showing the genetic organization of the invasion gene clusters from *S. typhimurium* and *S. flexneri*. The relative positions of each gene are indication and the directions of gene  
35 transcription are indicated by arrows. Arrows are not

- 18 -

drawn to scale. Gene clusters conserved in sequence and gene order are indicated by stippling (*inv-spa/mxi-spa*), crosshatching (*prglJK/mxiHI*), and dark arrows (*ssp/ipa*). Genes with no homologues within the respective regions are shown as open arrows.

Fig. 19 is a depiction of the nucleic acid sequence of *SspB* (missing part of the 5' end) (SEQ ID NO: 1).

Fig. 20 is a depiction of the nucleic acid sequence of *SspC* (SEQ ID NO: 2).

Fig. 21 is a depiction of the nucleic acid sequence of *SspD* (SEQ ID NO: 3).

Fig. 22 is a depiction of the nucleic acid sequence of *SspB* (missing part of the 3' end) (SEQ ID NO: 4) and the predicted amino acid sequence *SspB* (partial c-terminal) (SEQ ID NO: 5).

Fig. 23 is a depiction of the predicted amino acid sequences of *SspC* (SEQ ID NO: 6), *SspD* (SEQ ID NO: 7), and *SspA* (partial amino terminal) (SEQ ID NO: 8).

Fig. 24 is a depiction of the nucleic acid sequences of *iagB* (SEQ ID NO: 9) and *stpA* (SEQ ID NO: 10).

Fig. 25 is a depiction of the predicted amino acid sequences of *iagB* (SEQ ID NO: 11) and *stpA* (SEQ ID NO: 12).

Fig. 26 is a depiction of the nucleic acid sequence of *prgH* (SEQ ID NO: 13).

Fig. 27 is a depiction of the predicted amino acid sequences of *prgB* (SEQ ID NO: 14).

Fig. 28 is a depiction of the nucleic acid sequence of *SspBCDA* (truncated at 3' and 5' ends) (SEQ ID NO: 15).

Fig. 29 is a depiction of the nucleic acid sequence of *prgH* and 5' and 3' flanking sequences (SEQ ID NO: 16).

- 19 -

Ssp Proteins and Genes

The *Salmonella* secreted proteins (Ssp) of the invention have a variety of uses. For example, they can be used as diagnostic reagents, therapeutic agents, and  
5 research products. The genes encoding Ssp also have a variety of uses. For example, they can be used as diagnostic reagents. They can also be used to create vaccines including live attenuated vaccines.

Because *Salmonella* infection is a significant  
10 health problem and because Ssp proteins are soluble proteins that are found on the surface of *Salmonella*, various Ssp, DNA encoding various Ssp, and antibodies directed against various Ssp are useful in diagnostic assays. Because Ssp are required for optimal virulence,  
15 DNA encoding a mutant Ssp having decreased function can be used to create strains of *Salmonella* with reduced virulence. Such strains are useful as live vaccines.

An Ssp (or a portion thereof which can gain entry into the cytoplasm) can be used to translocate a second  
20 molecule, e.g., a polypeptide, into the cytoplasm of a cell. This approach can be useful for the induction or priming of cytotoxic lymphocytes (CTL) directed against the second molecule. An Ssp (or a portion thereof capable of translocating an attached second molecule) can  
25 be used to introduce a second molecule into the cell cytoplasm for the purpose of drug delivery. Often the second molecule is a polypeptide which is covalently linked to an Ssp (or a portion thereof), e.g., by a peptide bond. Such molecules can be readily produced  
30 first preparing a chimeric gene encoding the Ssp (or portion thereof) and the second molecule as a single polypeptide chain. This gene can be used to prepare the fusion protein for administration to a patient. Alternatively, the chimeric gene can be introduced into a

- 20 -

strain of *Salmonella* which can then be used as either a live vaccine or drug delivery system.

#### Ssp as Diagnostic Reagents

An Ssp can be used as a diagnostic tool for the  
5 detection of *Salmonella* infection in a patient or to evaluate status of an immune response to *Salmonella*. For example, one or more Ssp can be used an antigen in an ELISA assay to detect the presence of *Salmonella*-specific antibodies in a bodily fluid, e.g., blood or plasma,  
10 obtained from an infected patient or an individual suspected of being infected with *Salmonella*. Ssp can also be used to test immune cell activation, e.g., T or B cell proliferation or cytokine production, in a sample of patient-derived cells, e.g., peripheral blood mononuclear  
15 cells, to detect the presence of a cellular immune response to *Salmonella*.

Polynucleic acids (e.g., primers and probes) encoding all or part of an Ssp can be used in hybridization assays to detect the presence *Salmonella*  
20 infection, e.g., using a PCR assay or other probe or primer based assay designed to detect particular DNA sequences.

Antibodies capable of selectively binding a particular Ssp can be used to detect the presence of  
25 *Salmonella* in a biological sample. Such antibodies can be produced using standard methods.

#### Therapeutic Applications of Ssp Fusion Proteins

Fusion proteins comprising all or part of an Ssp and a second protein or polypeptide are useful for a  
30 variety of therapeutic applications such as vaccines (e.g., recombinant *Salmonella* vaccines or vaccines against heterologous pathogens), cell targeting agents for delivery of drugs (e.g., cytotoxic agents), and adjuvants, (e.g., to boost an immune response to a co-  
35 administered antigen).

- 21 -

To produce a recombinant *Salmonella* vaccine, a gene encoding an Ssp fusion protein can be introduced into a *Salmonella* vaccine. Because Ssp are involved in bacterial mediated endocytosis, the Ssp fusion protein will cause the second polypeptide or protein to be internalized by epithelial cells (or other cells to which the Ssp binds) of the individual to which the vaccine is administered. This internalization can trigger a Type I MHC-mediated response to the second protein or polypeptide. The induction of this response will lead to the induction of CTL (or the priming of CTL) specific for the second protein or polypeptide. The induction or priming of antigen-specific CTL can provide therapeutic or prophylactic benefits.

Purified fusion proteins can be used as recombinant vaccines. Proteins fused to Ssp are specifically targeted to epithelial cells or other cell types to which the Ssp bind; the fusion proteins are then internalized by the targeted cells. Thus, Ssp fusion proteins are useful to generate an immune response to the antigen to which the Ssp is linked or to deliver a therapeutic compound, e.g., a toxin for the treatment of cancer or autoimmune diseases in which the killing of specific cells, i.e., the cells to which a Ssp binds, is desired. Delivery of a toxin linked to a SspC or SspD polypeptide is especially useful in cancer therapy because many types of cancers are of epithelial cell origin.

Ssp fusion proteins which contain all or part of a Ssp linked to a heterologous protein can be made using methods known in the art. Two or more polypeptides may be linked together via a covalent or non-covalent bond, or both. Non-covalent interactions can be ionic, hydrophobic, or hydrophilic.

- 22 -

A covalent linkage may take the form of a disulfide bond. For example, the DNA encoding one of the polypeptides can be engineered to contain a unique cysteine codon. The second polypeptide can be  
5 derivatized with a sulfhydryl group reactive with the cysteine of the first component. Alternatively, a sulfhydryl group, either by itself or as part of a cysteine residue, can be introduced using solid phase polypeptide techniques.

10 A number of other covalent crosslinking agents, e.g., photoreactive crosslinkers, water-soluble crosslinkers, which are commercially available may be used to join a heterologous polypeptide to a Ssp to create a fusion protein. If the fusion protein is  
15 produced by expression of fused genes, a peptide bond serves as the link between the components of the fusion protein. Such fusion proteins are produced by expression of a chimeric gene in which sequences encoding all or part of an Ssp are in frame with sequences encoding the  
20 second protein or polypeptide. In some circumstances it may be useful to include a linker polypeptide between the Ssp and second protein of polypeptide.

Internalization of the fusion protein may not require the presence of a complete Ssp protein. A  
25 internalization-competent portion of an Ssp will be adequate in many circumstances. Whether a particular portion of a selected Ssp is sufficient for internalization can be tested as follows. The selected portion of an Ssp is fused to a calmodulin-dependent  
30 adenylate cyclase. If this test fusion protein is internalized, it will be exposed to calmodulin and the cyclase will be activated. The presence of adenylate cyclase activity can then be used as a measure of internalization. This general approach is described by  
35 Sorg et al. (*Molecular Microbiol.* 14:583-94, 1994).



- 23 -

Ssp are virulence factors that alter the ability of bacteria to be internalized by specific populations of host cells and to induce an immune response. *Salmonella* with mutations in genes encoding Ssp are useful in the manufacture of live *Salmonella* vaccines with altered cell tropism.

Deletion or overexpression of Ssp in *Salmonella* can be used to target strains or fusion proteins to various mammalian cell types. Invasion of epithelial cells or macrophages can be selected depending on the Ssp mutated. For example, use of *Salmonella* as an antigen or drug delivery vehicle can be optimized by deleting part or all of a gene encoding a Ssp involved in bacterial mediated endocytosis (or mutating such a gene to impair Ssp function), thereby minimizing the ability of *Salmonella* to invade epithelial cells (and therefor maximizing antigen delivery to antigen presenting cells such as macrophages). In this manner, strains with mutated Ssp genes can be used to modulate the host immune system. Deletion of Ssp genes in *Salmonella* can also be used to alter the ability of *Salmonella* to stimulate IL-8 secretion by epithelial cells.

Fusions of antigens to Ssp genes can be used to facilitate an immune response to the linked antigens for the purpose of generating an antigen-specific cytotoxic T cell response in a patient. For example, Ssp fusions to viral antigens are useful as therapeutic vaccines for diseases such as AIDS and *Herpes genitalis* in which the generation of a cytotoxic T cell (CTL) response is desired. Delivery of antigens in this manner favors the generation of an antigen-specific CTL response because the Ssp portion of Ssp fusion protein mediates translocation of the fusion protein across eucaryotic cell membranes into the intracellular compartments in the cytoplasm of cells which participate in class I MHC-mediated

- 24 -

antigen processing and presentation, i.e., the generation of class I MHC-restricted antigen-specific CTLs.

Fusion proteins which include all or part of a Ssp linked to a cytotoxic molecule can be used to target a  
5 cytotoxic molecule to a specific cell type, e.g., an epithelial cell-derived cancer cell, which would then be killed by the cytotoxic agent. Cytotoxic fusion proteins can be synthetically or recombinantly produced and administered directly to a patient. Alternatively, live  
10 *Salmonella* expressing a cytotoxic Ssp fusion protein can be administered and allowed to produce and secrete the fusion protein *in vivo*.

Ssp are also useful as adjuvants to boost the immunogenicity of antigens with which they are delivered  
15 or to which they are chemically or recombinantly linked. Ssp that have enzymatic effects, e.g., phosphatase activity, on certain types of eucaryotic cells can be used to promote specific types of immune responses such as TH2 or TH1 T cell responses. Since these proteins are  
20 secreted and are likely taken up in the cytoplasm of eucaryotic cells, gene fusions to these proteins are likely to be more immunogenic and more efficient in inducing the development of an immune response, particularly a class I MHC-restricted CTL response.

25 Various oral and parenteral delivery systems are known in the art and can be used to deliver the Ssp polypeptides and/or chimeric antigens of the invention, such as encapsulation in liposomes, or controlled release devices. The compositions of the invention can be  
30 formulated in a pharmaceutical excipient in the range of approximately 10  $\mu$ g/kg and 10 mg/kg body weight..

The compositions and methods of the invention provide the tools with which to construct better vaccines against *Salmonella* infection and for the prevention and  
35 treatment of other diseases, e.g., cancer and AIDS, by

- 25 -

using *Salmonella* secreted proteins as carriers of heterologous antigens, e.g., tumor antigens or viral antigens, either as purified components or as hybrid proteins produced in live *Salmonella* vaccine strains.

#### 5 Ssp and Attenuated Bacterial Strains

Deletion or mutation of one or more Ssp genes can be used to attenuate vaccine strains. For instance deletion of Ssp genes leads to lack of neutrophil transmigration across epithelial cell

10 monolayers (a model system that correlates well with the ability of certain strains to cause gastroenteritis).

Vaccine strains are usually administered at doses of  $1 \times 10^5$  to  $1 \times 10^{10}$  cfu/single oral dose. Those skilled in the art can determine the correct dosage using

15 standard techniques.

#### Research products

Ssp with enzymatic activity, e.g., *Salmonella* tyrosine phosphatase (stpA), can be used as reagents for protein modification. StpA catalyzes the release of  
20 phosphate groups from tyrosine residues in proteins, and thus, is especially useful in the field of signal transduction. Since a number of eucaryotic and procaryotic signal transduction proteins are regulated by the phosphorylation and dephosphorylation of tyrosine  
25 residues, stp can be used to deactivate or activate these proteins, thereby altering intracellular signal transduction. Thus, Stp can be used as a research tool to study and evaluate phosphorylation-regulated signal transduction pathways.

#### 30 Modification of Ssp and Ssp Variants

When an Ssp is being used to translocate a second molecule into a eukaryotic cell, it may be useful increase expression of the Ssp (or Ssp fusion protein) so that BME is increased. Increased expression of *sspC*,  
35 *sspD* and other *ssp* genes may be accomplished using

- 26 -

methods known in the art, e.g., by introducing multiple copies of the gene(s) into the bacterial cell or cloning the Ssp-encoding DNA under the control of a strong promoter.

5 Under other circumstances it may be desirable to increase uptake of a bacterial strain, e.g., a *Salmonella* strain, by a macrophage in a mammal by impairing the normal invasion mechanism of the strain. This can be accomplished by decreasing expression of the DNA encoding  
10 the SspC and/or SspD (and thereby decreasing secretion of Ssp and/or SspD polypeptides) and administering the cell to the mammal. Ssp expression may be reduced using methods known in the art, e.g., insertion of a transposon (Tn) into the gene, deletion of some or all of the gene,  
15 mutating a gene upon which SspC and/or SspD expression depends, e.g., *prgH*, e.g., a deletion or Tn insertion in the *prgHIJK* operon. Instead of decreasing the expression of *sspC* and/or *sspD*, the method may include the step of impairing the function of one or both of the gene  
20 products, e.g., by Tn insertion, deletion mutagenesis, or by impairing the secretory pathway by which the gene products are secreted such that the gene products are produced but not effectively transported to the extracellular environment.

25 Example 1: PhoP/PhoQ Transcriptional Repression of *S. typhimurium* Invasion Genes: Evidence for a Role in Protein Secretion

The PhoP-repressed *prgH* locus of *S. typhimurium* may be important for signaling epithelial cells to  
30 endocytose *S. typhimurium*. The following series of experiments demonstrate that the *prgH* locus is an operon of four genes encoding polypeptides of 392 amino acids (*prgH*), 80 amino acids (*prgI*), 101 amino acids (*prgJ*), and 252 amino acids (*prgK*). These experiments also

- 27 -

demonstrate that expression of the 2.6-kb *prgHIJK* transcript is reduced when PhoP/PhoQ is activated, suggesting that PhoP/PhoQ regulates *prgHIJK* by transcriptional repression. Further, analysis of the culture supernatants from wild-type *S. typhimurium* revealed the presence of at least 25 polypeptides larger than 14 kDa. Additional experiments demonstrated that *prgH1::TnpH*, *phoP* constitutive (*PhoP<sup>C</sup>*), and *hil* deletion mutants have significantly defective supernatant protein profiles. A further set of experiments described below demonstrate that both the invasion and supernatant protein profile defects of the *prgH1::TnpH* mutant can be complemented by a 5.1 kb plasmid that included *prgHIJK*. Taken together these results suggest that PhoP/PhoQ regulates extracellular transport of proteins by transcriptional repression of secretion determinants and that secreted proteins are likely involved in signaling epithelial cells to endocytose bacteria.

The following reagents and procedures were used to evaluate the *prgH* locus.

#### Bacterial Strains, Growth and Conditions

*S. typhimurium* strain ATCC 14028s (American Type Culture Collection, Bethesda, MD) is a virulent wild-type parent strain from which all other *Salmonella* strains described in Example 1 were derived. Bacterial strains and plasmids are described in Table 1. Luria-Bertani broth (LB) was used as rich bacterial growth medium. Antibiotics were added to LB broth or agar in the following concentrations: ampicillin, 25 µg/ml; chloramphenicol, 50 µg/ml; kanamycin, 45 µg/ml.

#### DNA sequencing and analysis

Double-strand templates were sequenced by the dideoxy-chain termination method known in the art as modified for use with Sequenase<sup>TM</sup> (US Biochemicals, Corp.) and [ $\alpha$ -<sup>35</sup>S]dATP. Computer analysis of the DNA sequence

- 28 -

was accomplished with the GENEPRO (Riverside Scientific, Riverside, CA) and Wisconsin package (GCG, version 7) programs. The nucleotide sequence of the *prgHIJK* locus has been deposited in GeneBank under accession number

5 U21676.

RNA extraction, RNA blot analyses, and primer extension analyses

RNA was isolated from mid-log phase cultures ( $OD_{600} = 0.5$ ) of aerobically-grown (with shaking) and

10 microaerophically-grown (without shaking) *Salmonella* strains using a standard hot phenol procedure (Pulkkinen et al., *J. Bacteriol.* 173:86-93, 1993). For RNA blots, 20  $\mu$ g of RNA was diluted in  $H_2O$  and incubated for 15 minutes at 55°C in 50% formamide, 17.5% formaldehyde in 1

15  $\times$  Northern buffer (0.36 M  $Na_2HPO_4 \cdot 7H_2O$ , 0.04 M  $NaH_2PO_4 \cdot H_2O$ ). Samples were run on 1% agarose gels containing 6% formaldehyde and 1  $\times$  Northern buffer and were transferred to Gene Screen Plus membranes (NEN/Dupont). RNA was crosslinked to the membrane using

20 a Stratalinker<sup>TM</sup> UV crosslinker (Stratagene, La Jolla, CA). Membranes were hybridized and washed according to the manufacturer's protocol.

The DNA probes for RNA-DNA and DNA-DNA blot hybridization were obtained from recombinant plasmid DNA

25 by restriction endonuclease digestion or by polymerase chain reaction (PCR) using the GeneAmp<sup>TM</sup> PCR kit (Perkin-Elmer/Cetus). The following DNA probes were synthesized: a 841-bp *prgH* probe from the oligonucleotide primers IB07 (5'-CCAGGTGGATACGGA-3'; SEQ ID NO: 17;

30 nucleotides 1198 to 1212) and IB19 (5'-TAGCGTCCTCCCCATGTGCG-3'; SEQ ID NO: 18; nucleotides 2039 to 2021); a 433-bp *prgI-prgJ* probe from the primers IB26 (5'-CCGGCGCTACTGGCGGCG-3'; SEQ ID NO: 19), nucleotides 2304 to 2321) and DP04

35 (5'-AGCGTTTCAACAGCCCCG-3'; SEQ ID NO: 20), nucleotides

- 29 -

2737 to 2719); a 341-bp *prgK* probe from primers DP03 (5'-CGGGGCTGTTGAAACGC-3'; SEQ ID NO: 21), nucleotides 2720 to 2736) and DP08 (5'-AACCTGGCCTTTTCAG-3'; SEQ ID NO: 22), nucleotides 3060 to 3045); a 724-bp *org* probe from primers DP15 (5'-GGCAGGGAGCCTTGCTTGG-3'; SEQ ID NO: 23), nucleotides 3774 to 3792) and DP17 (5'-GTGCCTGGCCAGTTCTCCA-3'; SEQ ID NO: 24); and a 608-bp *pagC* probe from a *Psi* and *StuI* restriction-endonuclease digest of pWPL4 that contains the wild-type *pagC* gene.

10 DNA probes were radiolabelled using a standard method of random priming with [ $\alpha$ -<sup>32</sup>P]dCTP.

For primer extension analyses, oligonucleotide primers (0.2 picomoles) were end-labelled with [ $\gamma$ -<sup>32</sup>P]dATP (NEN/Dupont), annealed to *S. typhimurium* RNA (20 $\mu$ g) and

15 extended with reverse transcriptase (Gibco BRL, St. Louis, MO). Reactions were electrophoresed in 6% polyacrylamide, 8 M urea gels adjacent to sequencing reactions initiated with primers used for cDNA synthesis.

DNA blot hybridization analysis

20 Chromosomal DNA was isolated, restriction endonuclease digested, size fractionated in agarose gels, and transferred to GeneScreen Plus membranes (NEN/Dupont). For dot blot hybridization experiments, high stringency hybridization was performed according to

25 standard methods at 65°C using radiolabelled probes.

Protein isolation and analysis

Bacteria were grown in LB, with shaking at 37°C. Bacterial cultures were chilled to 4°C and centrifuged at 154,000  $\times$  g for 1.7 hours. The supernatant was carefully

30 removed and trichloroacetic acid (TCA) was added to a final concentration of 10%. The precipitates were collected by centrifugation at 69,000  $\times$  g for 1 hour, rinsed with cold acetone, dried and stored at 4°C. The bacterial cell pellet was fractionated to obtain

35 periplasmic, cytoplasmic, and membrane fractions.

- 30 -

Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10-12% polyacrylamide (0.1 M Tris pH 8.45, 0.1% SDS) gel using a standard Tris-glycine buffer system or  
5 standard Tris-tricine buffer system. TCA precipitates were mixed with sample buffer (250 mM Tris pH 6.8, 2% SDS, 0.0025% bromophenol blue, 5.0%  $\beta$ -mercaptoethanol, 10% glycerol) and heated to 100°C for 5 minutes. Proteins were visualized by staining with Coomassie  
10 Brilliant Blue R-250.

#### Enzyme assays

Presence of the marker enzymes, alkaline phosphatase (periplasm) and  $\beta$ -galactosidase (cytoplasm) were used to assess fraction purity. A plasmid, pPOS3,  
15 containing an arabinose-inducible *phoA* gene, was inserted into wild-type strain 14028s by transformation and moved into other strains using P22 bacteriophage-mediated transduction. Addition of arabinose (0.02%) to the culture medium induced transcription of the *phoA* gene.  
20 Determination of alkaline phosphatase activity of strains containing pPOS3 was performed using the substrate p-Nitrophenyl phosphate according to standard methods. The results were expressed in standard units for  $\beta$ -galactosidase (Miller, J.H., 1972, Experiments in  
25 Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 352-355).  $\beta$ -galactosidase was produced from a strain with a *mudJ*-generated gene fusion of *msg* and *lacZ*. The gene, *msg*, is constitutively expressed and not PhoP regulated.  $\beta$ -galactosidase  
30 activity of strains carrying *msg::MudJ* was measured using routine methods (Miller et al., supra).



- 31 -

Table 1. Bacterial strains, plasmids and relevant properties

<u><i>S. typhimurium</i></u>	<u>Relevant genotype</u>
ATCC 14028s	Wild Type
5 CS002	<i>pho-24</i>
CS019	<i>phoN2zxx::6251Tn10d-Cm</i>
IB040	CS019 with <i>prgH1::TnpHoA</i>
IB043	IB040 with pWKS5
CS015	<i>phoP-102::Tn10d-Cm</i>
10 CS451	14028s derivative of EE451 with $\Delta$ <i>h1</i>

*Escherichia coli*

DH5 $\alpha$  F<sup>-</sup> $\phi$ 8 $\phi$ dlacZ $\Delta$ M15 $\Delta$ (*lacZYA-argF*)U169endA1  
recA1hsdR17deoRthi-1supE44 $\lambda$ gyrA96relA1

Plasmids

- 15 pIB01: pUC19 (amp<sup>R</sup>) containing a 10.7-kb *EcoRV* fragment with *prgH1::TnpHoA* (kan<sup>R</sup>)
- pVB3 pUC19 containing a 5.9-kb *HindIII-EcoRI* fragment of the *prgH* locus
- 20 pWKS5 pWKS30 (amp<sup>R</sup>) containing a 5.1-kb *HindIII* fragment of *prgH* locus
- pWPL4 pUC19 containing a 5.0-kb *EcoRV* fragment of the *pagC* locus
- pPOS5 pBR322 containing arabinose-inducible *PhoA*

Cloning and sequencing of *prgH*

- 25 The DNA containing the *prgH1::TnpHoA* gene fusion was cloned based upon information derived from the physical map of restriction endonuclease sites surrounding the transposon insertion (Fig. 1) (Behlau et al., *J. Bacteriol.* 175:4475-4484, 1993, hereby
- 30 incorporated by reference). Chromosomal DNA from strain IB040 containing the *prgH1::TnpHoA* insertion was digested with the restriction endonuclease *EcoRV* and ligated into *SmaI* digested pUC19 to generate a library of recombinant plasmids. These recombinant plasmids were transformed
- 35 into *Escherichia coli* (*E. coli*) DH5 $\alpha$ . A recombinant plasmid containing a 10.7 kb *EcoRV* fragment was identified by selecting for kanamycin resistance (*TnpHoA*

- 32 -

encoded) and was designated pIB01 (Fig. 1). DNA hybridization analysis of strain IB040 with a radiolabelled 1.5-kb *HindIII*-*SacI*-generated DNA fragment of pIB01 resulted in hybridization to an approximately 5 10.7-kb *EcoRV* DNA fragment. This was approximately 7.7 kb (the size of *TnphoA*) larger than the 3-kb fragment present in the wild-type strain ATCC 14028s. This probe also hybridized strongly to plasmid pVB3 that contained the 5.9 kb *HindIII*-*EcoRI* fragment of the *hil* locus (Fig. 10 1), confirming the location of the *prgH* locus within this region. This data indicated DNA containing the *prgH1::TnphoA* insertion had been cloned.

The DNA sequence of the 4,034-bp *HindIII*-*SspI* fragment (within which the *TnphoA* insertion in *prgH* was 15 localized) was determined by sequencing plasmid pIB01 containing the cloned *prgH1::TnphoA* allele. This sequence was confirmed by DNA sequencing of pWKS5 containing the wild-type *prgH* allele (Fig. 1). Information from DNA sequence of the *prgH1::phoA* fusion 20 junction was used to determine the direction of transcription and correct reading frame of *prgH*. *TnphoA* was inserted after nucleotide 1548 within an open reading frame that extended from nucleotides 981 to 2156. *prgH* was predicted to encode a 392 amino acid polypeptide with 25 a calculated  $M_r$  of 44,459 daltons and pI of 5.86. The N-terminal portion of *prgH* was found to have a stretch of nonpolar residues followed by the motif Leu-Xaa-Gly-Cys at residues 24 to 27 (corresponding to nucleotides 1050 to 1061) characteristic of the processing site of 30 bacterial lipoproteins. There was a strong hydrophobic domain (amino-acid residue 144 to 154, corresponding to nucleotides 1410 to 1433) upstream of the *TnphoA* insertion.

Analysis of the nucleotide sequence located 35 upstream of *prgH* revealed an additional open reading

- 33 -

- frame from nucleotides 665 to 222, termed *orf1*, likely to be oppositely transcribed from *prgH*. The intergenic region between *orf1* and *prgH* was 216 nucleotides. *orf1* was predicted to encode a gene product of 148-amino-acid residues with a calculated  $M_r$  of 17,186. The start codon of *orf1* was preceded by a potential ribosome binding site at 7 to 11 nucleotides 5' to the predicted start of translation (5'-AAAGG-3', nucleotides 676 to 672) suggesting that this open reading frame was translated.
- 10 The *orf1* predicted gene product had no signal sequence nor any strong hydrophobic domains.

Identification of *prgI*, *prgJ*, and *prgK*

- Analysis of the nucleotide sequence located downstream from *prgH* revealed four additional open reading frames that were predicted to be transcribed in the same direction and form an operon: (a) nucleotides 2184 to 2423; (b) nucleotides 2445 to 2747; (c) nucleotides 2747 to 3502; and (d) nucleotide 3476 to beyond the 3' *SspI* site. The first three of these four open reading frames identified were designated *prgI*, *prgJ*, and *prgK* respectively. *prgI*, *prgJ*, and *prgK* were predicted to encode gene products of 80 amino acids ( $M_r$ , 8865 daltons), 101 amino acids ( $M_r$ , 10,929 daltons), and 252 amino acids ( $M_r$ , 28,210 daltons). The predicted gene products encoded by *prgI* and *prgJ* did not contain a signal sequence or strong hydrophobic domains. The predicted gene product encoded by *prgK* contained a N-terminal hydrophobic region followed by a potential lipoprotein processing site from amino-acid residue 15 to 18 (corresponding to nucleotides 2788 to 2800). The fourth open reading frame corresponded in DNA sequence to the *S. typhimurium* oxygen-regulated gene (*org*).

*prgH-K* transcription is negatively regulated by PhoP/PhoQ

- To determine whether *prgH* was negatively regulated by PhoP/PhoQ, RNA isolated from wild-type (ATCC 14028s)

- 34 -

and PhoP<sup>C</sup> (CS022) strains of *S. typhimurium* were analyzed. In numerous RNA blot analyses, the prgH-specific DNA probe hybridized with an approximately a 2600-nucleotide RNA from the wild-type strain (Fig. 2).

5 The size of the RNA that hybridized to the prgH probe was similar to that of the prgH-K open reading frame predicted from the DNA sequence (i.e., 2600 vs. 2522 nucleotides). In contrast, no transcript was seen when equal amounts and similar quality of RNA (as assessed by

10 methylene blue staining) isolated from the PhoP<sup>C</sup> strain was probed with prgH-specific DNA (Fig. 3). In comparison, when the same RNA preparations were hybridized with a pagC-specific probe, an approximately 1100-nucleotide pagC transcript was highly expressed in

15 the PhoP<sup>C</sup> strain (Fig. 2), consistent with the constitutive phenotype of pag gene expression in the PhoP<sup>C</sup> mutant (Pulkkinen et al., *J. Bacteriol.* 173:86-93, 1991, hereby incorporated by reference). These results indicate that regulation of prgH occurs at the level of

20 transcription.

Primer extension analysis was performed to obtain information on the possible initiation site of prgH transcription. Based on this analysis, the start of prgH transcription was predicted to begin approximately

25 32 nucleotides upstream from the prgH translational start (Fig. 3). Several different primers were used that resulted in primer extension products of differing lengths, but all predicted that transcription initiated at this site. The predicted -10 (5'-TAATCT-3') and -35

30 (5'-TTCATC-3') regions are similar to the consensus sequences for typical  $\alpha 70$  *E. coli* promoters. Similar to the results of RNA blot hybridization analysis, a primer extension product was detected only with RNA isolated from wild-type *S. typhimurium* and not with RNA isolated

35 from the PhoP<sup>C</sup> strain (Fig. 3).

- 35 -

The size of the RNA that hybridized to the *prgH*-specific probe suggested that *prgH-K* could form a transcriptional unit. Therefore, to determine whether *prgI-K* formed an operon that was regulated by PhoP/PhoQ, RNA blot hybridization and primer extension analysis were performed using DNA probes and primers specific to the *prgI*, *prgJ*, and *prgK* open reading frames. Similar to the results with *prgH*, the *prgI-J*- and *prgK*-specific DNA probes hybridized with an approximately 2600-nucleotide RNA isolated from wild-type *S. typhimurium* and not with RNA from the PhoP<sup>C</sup> strain (Fig. 2). No primer extension products less than 350 nucleotides were detected using RNA isolated from either the wild-type or PhoP<sup>C</sup> strains using *prgI*, *prgJ*, and *prgK* primers. These primers were from 1662 to 2332 nucleotides downstream from the predicted start of *prgH* transcription. These findings indicated that *prgH-K* were transcribed as an operon, heretofore referred to as *prgHIJK*. Furthermore, this operon was likely to be transcribed from the *prgH* promoter and was negatively regulated by PhoP at the level of transcription.

*org* is not regulated by PhoP/PhoQ

Although the above results suggested that the *prgHIJK* transcriptional unit did not include *org*, experiments were performed to test this possibility. Blot hybridization analysis was performed with RNA isolated from wild-type *S. typhimurium* and an *org*-specific DNA probe. As shown in Fig. 2, two distinct transcripts hybridized to the *org* probe: an approximately 1400-nucleotide abundant RNA and a minor RNA of approximately 3800 nucleotides. The size of the smaller RNA was similar to that of the *org* open reading frame (1400 vs. 1236 nucleotides). In comparison, only the major 1400-nucleotide RNA was seen when RNA from the PhoP<sup>C</sup> strain was hybridized with the *org*-specific DNA

- 36 -

probe, suggesting that the 3800-nucleotide RNA was PhoP repressed.

A minor RNA of approximately 3800 nucleotides also was detected in long exposure of wild-type RNA blots that were hybridized with either the *prgH*, *prgI-J*, or *prgK* probes, suggesting possible cotranscription of *prgHIJK* and *org*. However, both the major (1400 nucleotide) and minor (3800 nucleotide) transcripts were detected when RNA isolated from the *prgH1::TnphoA* strain was hybridized with the *org* probe, indicating that the *prgH1::TnphoA* insertion was not polar on either of the *org* transcripts. Because expression of an *org::lacZY* fusion was shown to be increased approximately fourteen fold in low-oxygen compared with high-oxygen tension, RNA from wild-type and PhoP<sup>C</sup> strains that were grown aerobically or microaerophically to an optical density at 260 nm of 0.5 were compared by blot hybridization with the *org*-specific DNA probe. No substantial difference was seen in the relative amounts of RNA transcripts detected in wild-type or PhoP<sup>C</sup> strains grown under these conditions. These data indicate that *org* did not form part of the *prgHIJK* operon and that it was not regulated by PhoP/PhoQ.

The *prgI*, *prgJ*, and *prgK* predicted polypeptides are similar to *S. flexneri* Mxi and *Y. enterocolitica* Ysc proteins

The sequences of the five predicted polypeptides (*PrgH*, *PrgI*, *PrgJ*, *PrgK*, and *Orf1*) were compared with the protein sequences translated from the GeneBank library using BLAST network software. This comparison revealed similarity between the predicted products of *prgI*, *prgJ*, and *prgK* and the *MxiH*, *MxiI*, and *MxiJ* proteins of *S. flexneri*. Each of these polypeptide sequences were similar over their entire length, with 65% (*PrgI* vs. *MxiH*), 38% (*PrgJ* vs. *MxiI*), and 46% (*PrgK* vs. *MxiJ*) of

- 37 -

positions occupied by identical residues (Figs. 4A-4C). The *prgI* and *prgK* predicted gene products were also similar to the YscF and YscJ proteins, respectively, of *Y. enterocolitica*, with 28% and 30% of positions occupied by identical residues. The Poisson probabilities were highly significant for each of these comparisons. No protein similar to the *prgH* or *orf1* predicted polypeptides was detected in the protein sequence library.

10 Isolation of proteins from *S. typhimurium* culture supernatants

The role of *prgHIJK* in *S. typhimurium* protein secretion was analyzed by examination of the proteins present in cell culture supernatant. Culture media of wild-type bacteria was collected for protein analysis by centrifuging stationary phase cultures at  $154,000 \times g$  for 1.7 hours. From 6-8  $\mu g/ml$  of protein was precipitated by addition of trichloroacetic acid (TCA) to overnight culture supernatants. The TCA-precipitable material in 20 ml of supernatant then was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5). Approximately 25 protein bands, ranging in molecular mass from 18-87 kDa, were detected by Coomassie brilliant blue staining.

25 To rule out the possibility that the supernatant protein bands represented proteins released from lysed cells, soluble and membrane fractions of whole cells and whole cell lysates were compared with proteins from the supernatant by SDS-PAGE (Fig. 5). Many of the major polypeptides in the supernatant (e.g., the polypeptide with molecular mass of 87 kDa) were not the major proteins in the other cellular fractions. Conversely, major intracellular soluble proteins and membrane proteins (e.g., the 36 kDa OmpC porin) were not detected 35 in the supernatant in this analysis. In addition,

- 38 -

following centrifugation, the overnight culture media from bacteria expressing alkaline phosphatase (a periplasmic protein) and  $\beta$ -galactosidase (a cytoplasmic protein) always contained less than 9% and 1%,

5 respectively, of the whole-cell activity of these enzymes. Although some of the supernatant protein bands may represent degradation products of larger protein species, these data indicate that *S. typhimurium* was capable of significant protein secretion.

10 To determine the timing of release of polypeptides in to the supernatant and to test for an effect of PhoP regulon mutations on secretion, supernatants from CS015, with a null mutation in PhoP (PhoP<sup>-</sup>), CS022 (PhoP<sup>C</sup>), and wild-type bacteria (ATCC 14208s) were compared. As shown  
15 in Fig. 6, the quantity of protein increased for each strain when supernatant samples taken from mid-log-phase (OD<sub>600</sub> = 0.6), late-log/early-stationary-phase (OD<sub>600</sub> = 1.1), and stationary-phase (OD<sub>600</sub> = 2.2) were compared. However, the pattern of major protein bands detected for  
20 each strain was unchanged from mid-log to stationary phase (Fig. 6).

Altered supernatant protein profiles of mutants defective in signaling epithelial cells

At each phase of growth examined, a similar  
25 pattern and quantity of protein was detected in the culture supernatants of PhoP<sup>-</sup> strain CS015 and wild-type bacteria (Fig. 6). In contrast, the protein level of 2 ml of PhoP<sup>C</sup> strain CS022 supernatant was 24% of wild type levels. At least 10 major protein bands seen in the  
30 wild-type supernatant were greatly reduced or undetectable by Coomassie blue staining of the CS022 supernatant, especially those of higher molecular weight (Fig. 6). In addition, four major protein bands appeared to be increased in amount in CS022 compared with  
35 wild-type supernatant (31.5 kDa, 30 kDa, 23 kDa, and 20



- 39 -

kDa) (Fig. 6). Although this result could be due to degradation of higher molecular weight polypeptides, these data suggest that the PhoP<sup>C</sup> mutant likely was defective in synthesis or secretion of Ssp.

5           The defect observed with the PhoP<sup>C</sup> mutant was consistent with *prg* gene products having a role in protein secretion. Therefore, the Ssp of strains having transposon insertion or deletion of *prgHIJK* were compared to wild-type bacteria (ATCC 14028s) by SDS-PAGE. As  
10 observed for the PhoP<sup>C</sup> mutant, IB040 (*prgH1::TnpHoA*) and CS451, containing a 10-kb deletion of *hil* locus ( $\Delta$ *hil*) DNA, each had a pronounced defect in their Ssp profile compared with the wild-type strain (Fig. 6 and 7). IB043 and CS451 culture supernatants contained 100% and 62%,  
15 respectively, of wild-type protein levels. At least 5 and 11 major protein bands seen in the wild-type supernatant were greatly reduced or undetectable by Coomassie blue staining of the IB040 and CS451, respectively. Five protein bands [87 kDa, 65 kDa, and  
20 three in the 35-40 kDa range (Fig. 7), two of which run as a doublet under different electrophoretic conditions (Fig. 6)] were undetectable in the supernatants of CS022, IB040, and CS451. These findings indicated that the presence of at least some of the products of the *prgHIJK*  
25 operon were necessary for synthesis or secretion of Ssp.

          The defect in bacterial mediated endocytosis associated with *prgH1::TnpHoA* was complemented by a low-copy number plasmid, pWKSH5, containing a 5.1-kb fragment including *prgHIJK*, *org*, and *orf1*. Consistent  
30 with this observation, the *prgH1::TnpHoA* mutant carrying pWKSH5 (strain IB043) had a supernatant protein profile similar to that of wild type (Fig. 7). Of the five protein bands undetectable or greatly reduced in culture supernatants of *prgH1::TnpHoA*, each was detected in IB043  
35 and three of them were increased in amount (87 kDa, 65

- 40 -

kDa, and 35 kDa) compared with wild-type supernatants. This finding demonstrates a correlation between the ability to secrete proteins and induction of epithelial cell bacterial mediated endocytosis.

5 The *prgH* locus is important for *S. typhimurium* to induce endocytosis by epithelial cells

The defect in BME of the *prgH1::TnphoA* mutant is complemented by a plasmid containing 5.1 kb of DNA from this region, indicating that the gene or genes disrupted  
10 by the *prgH1::TnphoA* insertion are important for BME.

Analysis of the DNA sequence of this region identified six potential open reading frames that could be affected by this transposon insertion. As depicted in Fig. 1, five of these open reading frames, namely those  
15 designated *prgH-K* are either disrupted (i.e., *prgH*) or are 3' to the *prgH1::TnphoA* insertion. The *orf1* translational start is 884 nucleotides upstream from the *TnphoA* insertion and that *orf1* is predicted to be oppositely transcribed from the *prgHIJK* operon.

20 An approximately 2600 nucleotide PhoP-repressed transcript was detected when RNA was hybridized with *prgH*-, *prgI-J*-, or *prgK*-specific DNA probes. In contrast, the predominant transcripts detected with *org* was smaller (approximately 1400 nucleotides), was not  
25 altered in the *prgH1::TnphoA* mutant, and was not repressed by PhoP. Primer extension analysis of the potential start site of transcription, the size of the *prgHIJK* transcript, and the presence of a potential transcriptional terminator immediately downstream of *prgK*  
30 also were consistent with transcription terminating before *org*.

In addition to the major transcripts of *prgHIJK* and *org*, a minor PhoP-repressed transcript of approximately 3800 nucleotides also was detected in  
35 multiple RNA blots hybridized with the *org* and *prgH*,

- 41 -

*prgI-J*, or *prgK* DNA probes. This minor RNA was similar in size to the combined *prgHIJK* and *org* open reading frames (i.e., 3731 nucleotides) and, thus, could represent cotranscription of *prgHIJK* and *org*. However, 5 both the 3800- and 1400-nucleotide transcripts were detected in RNA from the *prgH1::TnphoA* mutant suggesting that the 3800-nucleotide RNA did not represent cotranscription of *prgHIJK* and *org*. These data indicate that one or more genes in the *prgHIJK* operon are 10 important to BME of epithelial cells.

A *PhoP* constitutive mutation repressed the synthesis of approximately 20 *prg*-encoded cell-associated protein species (Miller et al., *J. Bacteriol.* 172:2485-2490, 1990, herein incorporated by reference). Although 15 *PhoP/PhoQ* has been shown to transcriptionally activate *pag* (Miller et al., *Proc. Natl. Acad. Sci. USA* 86:5054-5058, 1989, herein incorporated by reference; Pulkkinen et al., *supra*, herein incorporated by reference), the mechanism of protein repression by *PhoP/PhoQ* had not been 20 characterized prior to the present studies. No transcript was detected when RNA from the *PhoP* constitutive mutant was probed with *prgH*-, *prgI-J*-, or *prgK*-specific DNA, indicating that the *prgHIJK* operon was negatively regulated by *PhoP/PhoQ* at the level of 25 transcription. Thus, *PhoP/PhoQ* can both activate and repress transcription of virulence genes.

Consistent with the role of one or more of the products of *prgHIJK* in bacterial mediated endocytosis and possibly in protein secretion, a low-copy plasmid 30 containing 5.1 kb of DNA (IB043), including *prgHIJK*, *org*, and *orf1*, complemented both the bacterial mediated endocytosis defect and the supernatant protein profile defect of the *prgH1::TnphoA* mutant. Based upon its similarity to *MxiJ* and *YscJ*, which are 35 membrane-associated lipoproteins that are necessary for

- 42 -

export and secretion of Ipa and Yops protein respectively, the *prgK* gene product is most likely to have such a role in bacterial mediated endocytosis and protein secretion. Similar to *PrgK*, *PrgH* was predicted to be a membrane lipoprotein. However, in contrast to *prgI-K*, which are similar to plasmid-encoded genes of *Shigella* and *Yersinia* spp., a *prgH* DNA probe hybridized to chromosomal DNA but not virulence-plasmid DNA from *Shigella* spp.

Neither they nor the *prgI* or *prgJ* predicted gene products have signal sequences or long hydrophobic domains that suggest their cellular localization. However, the location of these genes within operons that encode secretion determinants suggests that they may have a role in this process.

The predicted gene products of the *prgHIJK* operon were found to be similar to gene products required for protein secretion in other bacterial species. An analysis of proteins present in culture supernatants of *S. typhimurium* was performed. These experiments revealed that the supernatants of wild-type cultures contained a large number of protein bands, whereas strains with mutations affecting the *prgH* locus, including *prgH1::TnphoA*,  $\Delta$ *hil* and *PhoP<sup>C</sup>* were each defective in protein secretion as assessed by Ssp profiles. This analysis suggested that *PhoP/PhoQ* could control protein secretion, at least in part, by repressing *prgHIJK* whose products could form part of a secretion machinery. Furthermore, the finding that *PhoP<sup>C</sup>* and  $\Delta$ *hil* mutants were associated with greater defects in their Ssp profile compared with the *prgH1::TnphoA* mutant suggested that more than one mechanism may be involved in protein secretion and that gene products encoded by the 10 kb region that is deleted in the *hil* mutant also contribute to secretion of Ssp.

- 43 -

Since the strains with altered Ssp profiles were each impaired in signaling epithelial cells, these data suggest that Ssp are involved in signaling such cells to initiate BME. The finding that five Ssp were missing from culture supernatants of the *prgH* mutant suggested that one or more of these proteins were specifically involved in BME, e.g., Ssp and/or *prgHIJK* gene products may form a structure on the surface of *S. typhimurium* which induces bacterial mediated endocytosis.

10 *S. typhimurium* strains with transposons inserted between *prgH* and *spa* that result in reduced bacterial mediated endocytosis were also missing a subset of the Ssp missing from the *prgHIJK* mutant. DNA sequence analysis of the regions flanking the transposon  
15 insertions revealed deduced protein sequences that were similar to IpaB and IpaD of *S. flexneri*. These data suggest that the transposon insertions define an operon in *S. typhimurium* that encodes Ipa homologues.

Example 2: *Salmonella typhimurium* Secreted Invasion

20 Determinants

Two *Salmonella typhimurium* secreted protein (Ssp) mutants with transposon insertions located between *spaT* and *prgH* were identified. One mutant lacks the 87 kDa Ssp, while the other lacks Ssp of 87, 42, and 36 kDa.  
25 The invasiveness of these mutants implicates the 42 and 36 kDa Ssp, but not the 87 kDa Ssp in invasion. DNA sequencing of this region identified two complete and two partial open reading frames (designated *sspB*, *sspC*, *sspD*, and *sspA*).

30 The deduced amino acid sequences of *sspBCDA* are homologous to *Shigella flexneri* secreted proteins IpaB, IpaC, IpaD, and IpaA. Complementation analyses and amino-terminal sequencing showed that *sspC* and *sspA* encode the 42 kDa and the 87 kDa Ssp and that both

- 44 -

proteins are secreted without amino-terminal processing. SspA is abundantly secreted by wild type bacteria but is completely retained within the cellular fraction of a mutant in *prgHIJK* encoding part of the Ssp secretion apparatus. A precipitate containing SspC and three major Ssp of 63, 59, and 22 kDa was isolated from culture supernatants of wild type bacteria. These data indicate that major secreted invasion determinants of *S. typhimurium* are structurally and functionally homologous to *S. flexneri* Ipa proteins.

The following reagents and experimental procedures were used to characterize Ssp.

Construction of plasmids and strains:

To construct pCH002, pVV8-1 was cut with *EcoRI*, the 11 kb fragment eluted from a 1% agarose gel and cloned into the *EcoRI* site of pWSK29. In pCH002, transcription of *sspCDA* is driven from the *lac* promoter. pCH004 was constructed by cloning the 3 kb *BamHI* fragment from pCH002 into the *BamHI* site of pWSK29. pCH005 contains the 4 kb *EcoRI*-*PvuII* fragment from pCH002 cloned into *EcoRI*-*HincII* restricted pWSK29. pCH006 was constructed by restriction of pCH005 with *NcoI* and religation of the 1.7 kb and the 5 kb fragment. The correct orientations of the cloned inserts were confirmed by appropriate restriction analyses.

PCR of a chromosomal fragment of EE638 comprising the 5'-region of *Tn5lacZY* and adjacent DNA was performed in three independent experiments by using primers OL 1 (5' CGCGGATCCATTATGGGATGTATCGG 3'; SEQ ID NO: 25) and OL2 (5' CCGGCAGCAAAATGTTGCAG 3'; SEQ ID NO: 26). The 0.8 kb amplified DNA fragments were then restricted with *BamHI* and cloned into pWSK29 for sequencing. All three sequences were identical.

Strain VB122 (*hila::kan-112*) was constructed as follows: the mutation was originally constructed on a

- 45 -

plasmid by inserting a *kan* cassette (Pharmacia Biotech, Piscataway, NJ) in a *HincII* site in the 5' region of the *hila* coding sequence. The plasmid-encoded *hila::kan-112* mutation was recombined into the chromosome, and the  
5 chromosomal mutation was confirmed by PCR analysis.

Mutant EE633 (*lacZY4*) was isolated by screening for oxygen regulated gene fusions created by random *Tn5lacZY* insertions in *S. typhimurium* VV114 (*hila::kan-114*) and further selection for insertions  
10 linked to a *hila::kan-114* by P22 transduction into *S. typhimurium* SL1344 and selection for Tet<sup>R</sup> and Kan<sup>R</sup>.

Media and growth conditions for bacterial cultures:

Bacteria were grown in LB broth at 37°C. If  
15 necessary, selection was carried out using 50 µg/ml ampicillin, 10 µg/ml tetracycline, or 25 µg/ml kanamycin.

Preparation and analysis of *S. typhimurium* supernatant proteins:

Bacterial cultures were grown for 16 to 17 hours  
20 in 12 ml LB in 1.5 x 14 cm glass tubes at 37°C on a TC-7 roller (New Brunswick, Edison, NJ) at 50 rev./min. Soluble proteins from culture supernatants were obtained as described above. Precipitates in the culture were retrieved, rinsed 5 times with 1 ml H<sub>2</sub>O, dissolved in  
25 sample buffer (4% SDS, 12% glycerol, 5% β-mercaptoethanol, 0.05 M Tris-HCl pH 6.8, 0.01% bromphenol blue), and resolved in 10% polyacrylamide gels using SDS-PAGE and a Tris-Tricine buffer.

Immunoblotting:

30 Whole cell samples were prepared from overnight cultures using standard methods with the additional step of filtering the culture through a Whatman 1 qualitative paper filter (Whatman International, Maidstone, Kent, England) before centrifugation. The proteins were  
35 resolved by SDS-PAGE and transferred to nitrocellulose by

- 46 -

electroblotting using a conventional transfer buffer. Western blots were incubated with polyclonal rabbit serum prepared against the 87 kDa Ssp. The immunogen was purified by SDS-PAGE and injected into New Zealand White  
5 rabbits (Charles River, Wilmington, MA). Serum was collected after two booster injections and subsequently absorbed with an acetone powder prepared from *S. typhimurium* strain EE63. Horseradish peroxidase-labelled goat anti-rabbit antibodies were used to label the  
10 primary antibodies and were visualized using chemiluminescence (ECL, Amersham, International, Buckinghamshire, England)

Invasion assays:

Invasion of HEP-2 epithelial cells was carried out  
15 according to the method of Behlau et al. (*J. Bacteriol.* 175:4475-4484, 1993). To minimize epithelial cell detachment from the bottom of the assay wells after bacterial uptake, the following modifications were introduced: invasion time was reduced from 90 to 60 min  
20 and gentamicin treatment was performed for 15 min with 100 µg/ml gentamicin, conditions which were shown to kill 99% of a bacterial culture of  $2 \times 10^8$  cells/ml.

N-terminal protein sequencing:

Proteins separated by SDS-PAGE were blotted on  
25 PVDF membranes (Bio-Rad, Hercules, CA) and stained with Ponceau-S. Blotted proteins were sequenced using an ABI 470A protein sequencer with 120A PTH-AA analyzer.



- 47 -

Table 7: Strains and plasmids used in this example

<u>Bacterial strain</u>	<u>Marker</u>
<i>E. coli</i> DH5 $\alpha$	F- $\phi$ 80dlacZ $\Delta$ M15 $\Delta$ (lacZYA-argF)U169endA1 recA1hsdR17(r <sub>K</sub> <sup>-</sup> , 5 m <sub>K</sub> <sup>+</sup> )deoRthi-lsupE44 $\lambda$ gyrA96relA1
<i>S. typhimurium</i> SL1344	wild type
VV114	h1l::kan-114
VB122	Kan <sup>R</sup> , h1lA::kan-112
EE637	Tet <sup>R</sup> , invF::lacZY11-5
10 EE633	Tet <sup>R</sup> , sspA::lacZY4
EE638	Tet <sup>R</sup> , sspC::lacZY11-6
<i>S. typhimurium</i> (ATCC14028s)	wild type
CS451	14028sah1l::Tn5-428
15 CS022	pho-24 (PhoP <sup>C</sup> )
IB04	prgH1::TnphoA
<u>Plasmid</u>	<u>Marker</u>
pWSK29	Amp <sup>R</sup>
pVV8-1	Tet <sup>R</sup>
20 pVV71	Amp <sup>R</sup>
pCH002	Amp <sup>R</sup> , sspCDA, h1lA
pCH004	Amp <sup>R</sup> , sspC
pCH005	Amp <sup>R</sup> , sspCD
pCH006	Amp <sup>R</sup> , sspD
25 <u>Identification of <i>S. typhimurium</i> Mutants with Transposon</u>	
<u>Insertions in Genes Encoding Ssp</u>	

To identify genes encoding Ssp, Tn5lacZY mutants of *S. typhimurium* SL1344 with transposon insertions located within the 40 kb "virulence island" (59-60 min. of the *S. typhimurium* chromosome) were analyzed for changed patterns in Ssp. An insertion in invF (invF::lacZY11-5), the first gene of the inv-spa operon, and a h1lA::kan-122 insertion in VB122 led to major defects in the pattern of Ssp similar to a mutation in the prgHIJK operon (prgH1::TnphoA) which has implicated in *S. typhimurium* protein secretion (see Example 1). Specifically, all three mutants lack 5 major Ssp of 36, 38, 42, 63 and 87 kDa, while the h1lA::kan-112 insertion leads to loss of some lower molecular weight protein bands in addition to these 5 Ssp (Fig. 8, lanes 4, 5, 6).

- 48 -

Two other mutants exhibited detectable loss of only one and of three Ssp, respectively. The supernatants from the mutant strain EE633 containing the fusion *lacZY4* were missing a protein of 87 kDa, while supernatants from the mutant strain EE638, containing fusion *lacZY11-6*, were missing protein species of 87, 42 and 36 kDa. In addition, supernatants from EE638 showed an increased abundance of a 63 kDa Ssp (Fig. 8, lanes 2, 3). *Tn5lacZY* in EE638 maps approximately 2.5 kb downstream from *spaT* while in EE633 the transposon maps 5.5 kb downstream from *spaT* as determined by Southern hybridization and PCR analyses. Both transposons were inserted in the same orientation (Fig. 9). A degenerate pool of oligonucleotides synthesized according to the sequence of the 12 amino-terminal amino acids of the 87 kDa protein (VTSVRTQPPVIM; SEQ ID NO: 27), hybridized specifically to a 5.5 kb *Bam*HI fragment in pVV71 which comprises sequences between *hila* and *spaT* (Fig. 9). These data indicate that the 87 kDa Ssp is encoded in the chromosomal region adjacent to the transposon insertions. *Tn5lacZY* in EE633 is likely to be directly within the gene encoding the 87 kDa Ssp, while *Tn5lacZY* in EE638 is likely to be inserted within one of the genes encoding the 42 and the 36 kDa Ssp having a polar effect on the synthesis of the other two Ssp missing in supernatants of this mutant.

#### Secretion of the 87 Ssp kDa Ssp is Dependent on *prgHIJK*

Since it was possible that the absence of the 87 kDa Ssp (Ssp87) in supernatants of EE633 and EE638 was due to impaired secretion rather than expression, whole cell lysates and supernatants of various strains were analyzed by immunoblotting with antiserum raised against partially purified Ssp87. Fig. 11 shows that Ssp87 of wild type *S. typhimurium* is found mainly in the supernatant, although some of the protein is detected in

- 49 -

the cellular fraction (Fig. 11, lane 1). In contrast to wild type bacteria, all of the protein is found in the cellular fraction of the *prgH1::TnpHoA* mutant IB040 (lane 3). Ssp87 could not be detected in the cellular fractions nor in supernatants of various invasion and secretion mutants: CS022 (PhoP<sup>C</sup>), a mutant which constitutively represses PhoP regulated genes (Miller et al., *J. Bacteriol.* 172:2485-2490, 1990, hereby incorporated by reference) (lane 2), CS451 (Δ*hil*::Tn5-428) carrying a 10 kb chromosomal deletion of the *hil* locus between 59 and 60 min. (lane 4), EE638 (*lacZY11-6*) (lane 5), and EE633 (*lacZY4*) (lane 6). The signal at 51 kDa in the supernatant fraction of wild type bacteria might represent a degradation product of Ssp87, while the faint band at 34 kDa is likely nonspecific hybridization since it is present in all bacteria analyzed. These results demonstrate that lack of Ssp87 in supernatants of EE633 and EE638 is due to impaired expression while lack of Ssp87 in supernatants of IB040 (*prgH1::TnpHoA*) is caused by impaired secretion of the protein. These results further show that expression of the gene encoding Ssp87 is affected by the *hil* deletion and that expression of Ssp87, either directly or indirectly, is repressed by PhoP.

25 Strain EE638, but not EE633, Is Markedly Deficient in Invasion

To determine the function of the 87, 42, and 36 kDa Ssp in invasion of epithelial cells, the ability of strain EE638 and EE633 to invade HEp-2 cells was analyzed. EE638 showed more than a 100-fold reduction in invasiveness when compared to wild type bacteria, while EE633 exhibited invasion levels comparable to wild type bacteria (Fig. 9). These results suggested that the 36 and/or the 42 kDa Ssp but not the 87 kDa Ssp are required for epithelial cell invasion. In addition, observation

- 50 -

of interactions between these mutants and PtK2 cells by time-lapse videomicroscopy indicated that the ability of EE638 to induce epithelial cell membrane ruffling is also markedly reduced, while EE633 induced localized membrane ruffles at a frequency similar to wild type *S. typhimurium*.

The Tn5lacZY Insertions in EE638 and EE633 Define a Chromosomal Region Encoding Ssp *S. typhimurium* Homologues of the *Shigella ipaBCDA* Operon

10 To determine the gene(s) affected by the transposon-insertions in EE638 and EE633, part of a 11 kb EcoRI subclone of pV8-1 was sequenced. Two complete and two partial open reading frames (ORFs), positioned in the same transcriptional direction, were identified (Fig. 9).  
15 The deduced gene products of the complete ORFs exhibit similarity to *Shigella* secreted proteins IpaC and IpaD (31% identity, 47% similarity; 37% identity, 56% similarity) respectively, and therefore were designated *sspC* and *sspD* (see Fig. 13 and Fig. 14). The gene  
20 products of the complete open reading frames were designated *sspC* and *sspD*. The amino acid sequence derived from the 5'-end of *sspC* was identical to the amino-terminal sequence of the 42 kDa Ssp (underlined in Fig. 13). The deduced gene product of the partial ORF  
25 located immediately upstream from *sspC* shows 47% identity (67% similarity) to the carboxyterminal portion of *S. flexneri* secreted protein IpaB and was designated *sspB* (Fig. 12). The ORF starting immediately downstream of *sspD* was designated *sspA*. The amino acid sequence  
30 deduced from the 5' end of an ORF starting immediately downstream from *sspD* did not exhibit similarity to IpaA. However, DNA sequencing of internal parts of the gene predicted that the protein encoded by this gene, designated *sspA*, is similar to IpaA. Nevertheless, the  
35 sequence of amino acids 2-13 (underlined in Fig. 15) was

- 51 -

identical to the amino-terminal sequence of the 87 kDa Ssp (see above). *sspB*, *sspC*, *sspD*, and *sspA* are separated by 27, 70, and 15 bp, respectively, and putative ribosome binding sites precede *sspC*, *sspD*, and *sspA*.

The amino acid similarities of Ssp to Ipa do not extend over the entire lengths of the proteins. The similarities between SspC/IpaC and SspD/IpaD are highest in the carboxy-terminal regions, while the central parts of SspB and IpaB are conserved (see Fig. 12, 13, and 14). These similarities could reflect conservation in regions of the proteins required for secretion and/or invasion. Although both SspC and SspD appear to be secreted by the same mechanism, no obvious similarities or motifs common to these proteins were detected, thus implying conformational rather than sequential features in the secretion of proteins by type III secretion pathways.

The precise insertion of Tn5lacZY in EE638 was determined by cloning and sequencing of a PCR product comprising the 5' region of the transposon and upstream chromosomal sequences and was shown to be located 189 bp downstream from the ATG start codon of *sspC*. The order of the *ssp* genes and the Ssp profile of EE638 indicate that the transposon insertion in *sspC* is polar on expression of *sspD* and *sspA* and that these genes are likely to be organized in a singly transcribed unit. Both *sspC* and *sspD* are Necessary for *S. typhimurium* Invasion of Epithelial Cells

A complementation analysis was carried out to determine the minimal fragment necessary for complementation of the epithelial cell invasion defect of EE638 (*sspC::lacZY11-6*) as well as for reconstitution of Ssp. All analyzed fragments were cloned downstream from the *lac* promoter in the 6-8 copies/chromosome vector pWSK29. As shown in Fig. 10, a 3.9 kb *EcoRI*-*PvuII*

- 52 -

fragment comprising *sspC* and *sspD* in pCH005 was sufficient to complement the invasion defect of EE638 to wild type levels. When analyzed for Ssp, EE638 [pCH005] showed a pattern of Ssp similar to the wild type strain [PWSK29] except for the missing 87 kDa protein (SspA) (Fig. 16, lane 4). EE638 transformed with pCH002 carrying an 11 kb *EcoRI* fragment was partially complemented for invasion as well as for all 3 missing Ssp (Fig. 10 and Fig. 16, lane 6). In contrast, EE638 transformed with plasmids that contained either *sspC* or *sspD* alone (pCH004 and pCH006, respectively) were not complemented for invasion but showed reconstitution of the 42 kDa Ssp (SspC) or the 36 kDa Ssp (SspD), respectively (Fig. 10 and Fig. 16, lanes 3 and 5). In addition, the abundance of a 63 kDa Ssp, which was found to be more abundant in supernatants of EE638, was reduced in supernatants of strains EE638 [pCH005], EE638 [pCH006], and EE638 [pCH002] and of SL1344 [pCH002]. These results demonstrate that both *SspC* and *SspD* are necessary for invasion of epithelial cells and indicate that *SspC* encodes the 42 kDa Ssp while the 36 kDa Ssp is likely to be encoded by *SspD*. In addition, complementation of the invasion defect of EE638 with pCH005 indicates that invasiveness is not influenced by the observed changes in the abundance of the 63 kDa Ssp.

A Precipitate Found in *S. typhimurium* Culture Supernatants Contains Highly Abundant SspC and Other Proteins

Supernatants from *S. typhimurium* wild type cultures contained a precipitate that, when solubilized in reducing SDS sample buffer, separates into at least four highly abundant protein bands of 63, 59, 42 and 22 kDa on SDS-PAGE (see Fig. 17, lane 1). Protein precipitates were also found in culture supernatants of EE638 and EE633, but not in supernatants of *S.*

- 53 -

*typhimurium* mutants with global defects in protein secretion [CS022 (Pho<sup>C</sup>), IB040 (*prgH::TnphoA*), CS451 ( $\Delta$ hil::Tn5-428) and VB122 (*hila::kan-112*). *S. typhimurium* 14028s, the wild type parent of CS022 and  
5 IB040, showed the same protein pattern of precipitated material as SL1344]. The precipitate from EE633 cultures showed a similar composition to that of wild type precipitate by SDS-PAGE analysis. In contrast, a major protein band of 42 kDa was absent from the precipitate  
10 isolated from cultures of EE638 (Fig. 17, lane 2). Amino-terminal sequencing of this 42 kDa Ssp identified it as encoded by *SspC*. The identity of the amino-terminal protein sequence (MLISNVGINPAAYLN; SEQ ID NO: 28) with the amino acid sequence derived from the  
15 5'-region of *SspC* (Fig. 13) shows that no amino-terminal processing of *SspC* occurs prior to its release into the supernatant.

SDS-PAGE analyses of precipitated material from culture supernatants of EE638 [pCH004 (*SspC*)] and EE638  
20 [pCH005 (*SspCD*)] showed a pattern similar to wild type [pWSK29] material (Fig. 17, lane 3 and 4), confirming that the respective plasmids complemented the mutant for secretion of *SspC*. Protein patterns of soluble Ssp and precipitates isolated from untransformed cultures of  
25 SL1344 or EE638 were identical to those shown in Fig. 16, 17, lane 1 and 2, respectively. Precipitate of EE638 [pCH006 (*SspD*)] was found to be similar to precipitate from EE638 [pWSK29] except for reduced abundance of a 63 kDa protein band (Fig. 17, lane 5). The precipitate from  
30 EE638 [pCH002 (*SspCDA*)] contained an additional major protein band of approximately 51 kDa, which was also present in precipitate from SL1344 [pCH002] (Fig. 17, lanes 6, 7). Comparison of precipitated proteins to soluble Ssp on SDS-PAGE (Fig. 17, lanes 8, 9) showed that  
35 *SspC* in the precipitate has the same electrophoretic

- 54 -

mobility as the 42 kDa soluble Ssp. These data suggest that the 42 kDa soluble Ssp is identical to precipitated SspC.

SspC and SspA are secreted proteins of 42 and 87 kDa, as demonstrated by amino-terminal sequencing and by complementation analyses. It is further likely that the 36 kDa protein encoded by *SspD* is secreted, since lack of a 36 kDa Ssp in supernatants of EE638 (*lacZY11-6*) was complemented by transformation of this mutant with plasmids containing *SspD*. The 63 kDa Ssp is the protein likely to be encoded by *SspB*.

SspA, SspB, SspC, and SspD appear to be targets of the *inv-spa-prgHIJK* encoded secretion apparatus, since these Ssp are missing in supernatants of mutants affecting expression or regulation of *inv-spa* and *prgHIJK* (Fig. 8). Typical for proteins secreted by type III secretion pathways, no amino-terminal processing of SspA and SspC was observed. The dependency of Ssp secretion on *prgHIJK* was further proven by demonstrating that SspA is abundantly secreted by wild type cells, while it is completely retained in the cellular fraction of the *prgH1::TnphoA* mutant IB040 (Fig. 11). The 38 kDa Ssp of the five major Ssp dependent on the *inv-spa-prgHIJK* secretion apparatus may be the product of the *invJ* invasion locus.

The immunoblot analysis of SspA secretion suggests that expression of the gene encoding SspA is negatively controlled by the virulence two component regulatory system PhoP/PhoQ. PhoP/Q has a global effect on protein secretion which is partially due to negative transcriptional regulation of *prgHIJK* (see Example 1).

The *SspBCDA* genes are located between the large *inv-spa* gene cluster and *prgHIJK* at 59 minutes on the *S. typhimurium* chromosome. Fig. 18 shows the relative positions of the invasion genes in *S. typhimurium* in comparison to their *S. flexneri* homologues, which are



- 55 -

clustered in a 31 kb region of a large virulence plasmid. The invasion genes cluster in three groups (*inv-spa/mxi-spa*, *Ssp/ipa*, and *prgIJK/mxiHIJ*) which exhibit conserved gene structure and organization, suggesting that these genes were acquired by horizontal gene transfer. Acquisition by horizontal gene transfer is further supported by the fact that these *S. typhimurium* invasion genes are within a 40 kb "virulence island" which, despite the otherwise high overall genetic similarity between *S. typhimurium* and *E. coli* K-12, is unique to *S. typhimurium*. However, the three invasion gene clusters from *S. flexneri* and *S. typhimurium* are in different relative positions to each other and are interspersed between non-homologous genes, thus implying multi-recombinational events in the evolution of these genetic regions.

In addition to soluble Ssp the supernatants of *S. typhimurium* cultures contained a flocculent precipitate consisting of SspC and three other major protein species of 63 (Ssp 63), 59 (Ssp 59), and 22 (Ssp 22) kDa. The combination and abundance of Ssp in the precipitate from *S. typhimurium* cultures is strikingly different from that in the soluble fraction (see Fig. 17). Though Ssp, including SspC, are found in both the precipitate and the soluble fraction, SspD, even when overproduced, was not detected in the precipitate. This emphasizes the difference in composition of precipitate and soluble fraction and supports the possibility of specific protein-protein interactions between the four Ssp leading to precipitate formation.

#### OTHER EMBODIMENTS

Using reagents derived from partial cDNA clones of an Ssp, e.g., SspA, the isolation of a full-length cDNA encoding the Ssp is well within the skill of those

- 56 -

skilled in the art of molecular biology. For example, a radiolabelled probe made from a known partial cDNA sequence can be used to identify and isolate from a library of recombinant plasmids cDNAs that contain regions with identical to the previously isolated cDNAs. The screening of cDNA libraries with radiolabelled cDNA probes is routine in the art of molecular biology (see Sambrook et al., 1989, *Molecular Cloning: a Laboratory Manual*, second edition., Cold Spring Harbor Press, Cold Spring Harbor, N.Y). The cDNA can be isolated and subcloned into a plasmid vector, and the plasmid DNA purified by standard techniques. The cDNA insert is sequenced using the dideoxy chain termination method well known in the art (Sambrook et al, *supra*).

Oligonucleotide primers corresponding to bordering vector regions as well as primers prepared from previously isolated cDNA clones can be employed to progressively determine the sequence of the entire gene.

Similar methods can be used to isolate Ssp which are related to SspA, SspB, SspC, or SspD. To isolate related Ssp, a probe having a sequence derived from (or identical to) all or a portion of SspA, SspB, SspC, or SspD can be used to screen a library of *Salmonella* DNA (or cDNA). DNA encoding a related Ssp will generally hybridize at greater stringency than DNA encoding other proteins. This approach can be used to identify *Salmonella typhimurium* Ssp as well as Ssp of other *Salmonella*.

#### Generation of Monoclonal Antibodies:

Monoclonal antibodies can be generated to purified native or recombinant gene products, e.g., Ssp, by standard procedures, e.g., those described in Coligan et al., eds., *Current Protocols in Immunology*, 1992, Greene Publishing Associates and Wiley-Interscience). To generate monoclonal antibodies, a mouse is immunized with

- 57 -

the recombinant protein, and antibody-secreting B cells isolated and immortalized with a non-secretory myeloma cell fusion partner. Hybridomas are then screened for production of specific antibodies and cloned to obtain a  
5 homogenous cell population which produces a monoclonal antibody. For example, hybridomas secreting the desired antibodies can be screened by ELISA. Specificities of the monoclonal antibodies can be determined by the use of different protein or peptide antigens in the ELISA.

10 Useful quantities of antibodies can be produced by either the generation of ascites fluid in mice or by large scale in vitro culture of the cloned antibody-producing hybridoma cell line. Antibodies can be purified by various chromatographic procedures known in the art, such  
15 as affinity chromatography on either immobilized Protein A or Protein G.

The invention also includes DNA encoding other Ssp (e.g., Ssp 54, Ssp 42, and Ssp 22) found in cell supernatants. Those skilled in the art can readily clone  
20 the corresponding genes based on the amino terminal sequence or the corresponding protein. The amino terminal sequence of Ssp54 is MNNLTLSXFXKVG (SEQ ID NO: 29). The amino terminal sequence of Ssp42 is MLISNVGINPAAYLN (SEQ ID NO: 30). The amino terminal  
25 sequence of Ssp 22 is TKITLSPQNFFI (SEQ ID NO: 31).

- 58 -

CLAIMS

1. Substantially pure DNA encoding a *Salmonella* secreted protein (Ssp).
2. The DNA of claim 1, wherein said DNA comprises  
5 the SspB gene.
3. The DNA of claim 2, wherein said DNA comprises the DNA sequence of SEQ ID NO: 1 or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 5.
4. The DNA of claim 1, wherein said DNA comprises  
10 the SspC gene.
5. The DNA of claim 4, wherein said DNA comprises the DNA sequence of SEQ ID NO: 2 or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 6.
6. The DNA of claim 1, wherein said DNA comprises  
15 the SspD gene.
7. The DNA of claim 6, wherein said DNA comprises the DNA sequence of SEQ ID NO: 3 or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 7.
8. The DNA of claim 1, wherein said DNA comprises  
20 the SspA gene.
9. The DNA of claim 8, wherein said DNA comprises the DNA sequence of SEQ ID NO: 4, or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 8.
10. The DNA of claim 1, wherein said DNA  
25 comprises the SspB gene, the SspC gene, the SspD gene and the SspA gene.

- 59 -

11. The DNA of claim 10, wherein said DNA comprises the DNA sequence of SEQ ID NO: 15.

12. The DNA of claim 1, wherein said DNA comprises the SspH gene.

5 13. The DNA of claim 12, wherein said DNA comprises the DNA sequence of SEQ ID NO: 13, or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 14.

10 14. The DNA of claim 1, wherein said DNA comprises the stpA gene.

15 15. The DNA of claim 14, wherein said DNA comprises the DNA sequence of SEQ ID NO: 10 or degenerate variants thereof encoding the amino acid sequence of SEQ ID NO: 12.

16 16. A cell which contains the DNA of claim 1.

17. A method of inducing uptake of a bacterial cell by an epithelial cell in a mammal, comprising increasing expression of the DNA of claim 4 or 6 in said cell and administering said cell to said mammal.

20 18. The method of claim 17, wherein said bacterial cell is a *Salmonella* cell.

25 19. A method of inducing uptake of a bacterial cell by a macrophage in a mammal, comprising decreasing expression of the DNA of claim 4 or 6 and administering said cell to said mammal.

20. A substantially pure SspC polypeptide.

- 60 -

21. The polypeptide of claim 20, comprising an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 6.

22. An active fragment of the polypeptide of  
5 claim 21.

23. A substantially pure SspD polypeptide.

24. The polypeptide of claim 23, comprising an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 7.

10 25. An active fragment of the polypeptide of claim 24.

26. A substantially pure SspH polypeptide.

27. The polypeptide of claim 26, comprising an amino acid sequence substantially identical to the amino  
15 acid sequence of SEQ ID NO: 14.

28. An active fragment of the polypeptide of claim 27.

29. A substantially pure IagB polypeptide.

30. The polypeptide of claim 29, comprising an  
20 amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO: 11.

31. An active fragment of the polypeptide of claim 40.

32. An antibody which binds to a Ssp.

- 61 -

33. A method of detecting a *Salmonella* infection in a mammal comprising contacting a biological sample derived from said mammal with the antibody of claim 32 and detecting the binding of said antibody to a Ssp in  
5 said sample, wherein said binding indicates that said mammal is infected with *Salmonella*.

34. A method of detecting the presence of *Salmonella* in a biological sample comprising contacting said sample with a Ssp-encoding DNA under high stringency  
10 conditions and detecting the hybridization of said DNA to nucleic acid in said sample, wherein hybridization indicates the presence of *Salmonella* in said biological sample.

35. A method of targeting an antigen to an  
15 epithelial cell in a mammal, comprising linking said antigen to an Ssp or active fragment thereof to produce a Ssp chimeric antigen and administering said chimeric antigen to said mammal.

36. The method of claim 35, wherein said Ssp is  
20 SspC or SspD.

37. A method of inducing a cytotoxic T cell immune response in a mammal, comprising linking said antigen to an Ssp or active fragment thereof to produce a Ssp chimeric antigen and contacting an antigen-presenting  
25 cell with said chimeric antigen.

38. A vaccine comprising a bacterial cell the virulence of which is attenuated by decreased secretion of a Ssp.

-62-

39. The vaccine of claim 38, wherein said bacterial cell is a *Salmonella typhimurium* cell.

40. The vaccine of claim 39, wherein said bacterial cell is a *Salmonella enteriditis* cell.

5 41. The vaccine of claim 38, wherein said bacterial cell is a *Salmonella typhi* cell.

42. A live *Salmonella* cell in which a gene encoding a heterologous antigen is inserted into a Ssp-encoding gene.

10 43. A method of vaccinating an animal against a *Salmonella* infection comprising administering the vaccine of claim 38.

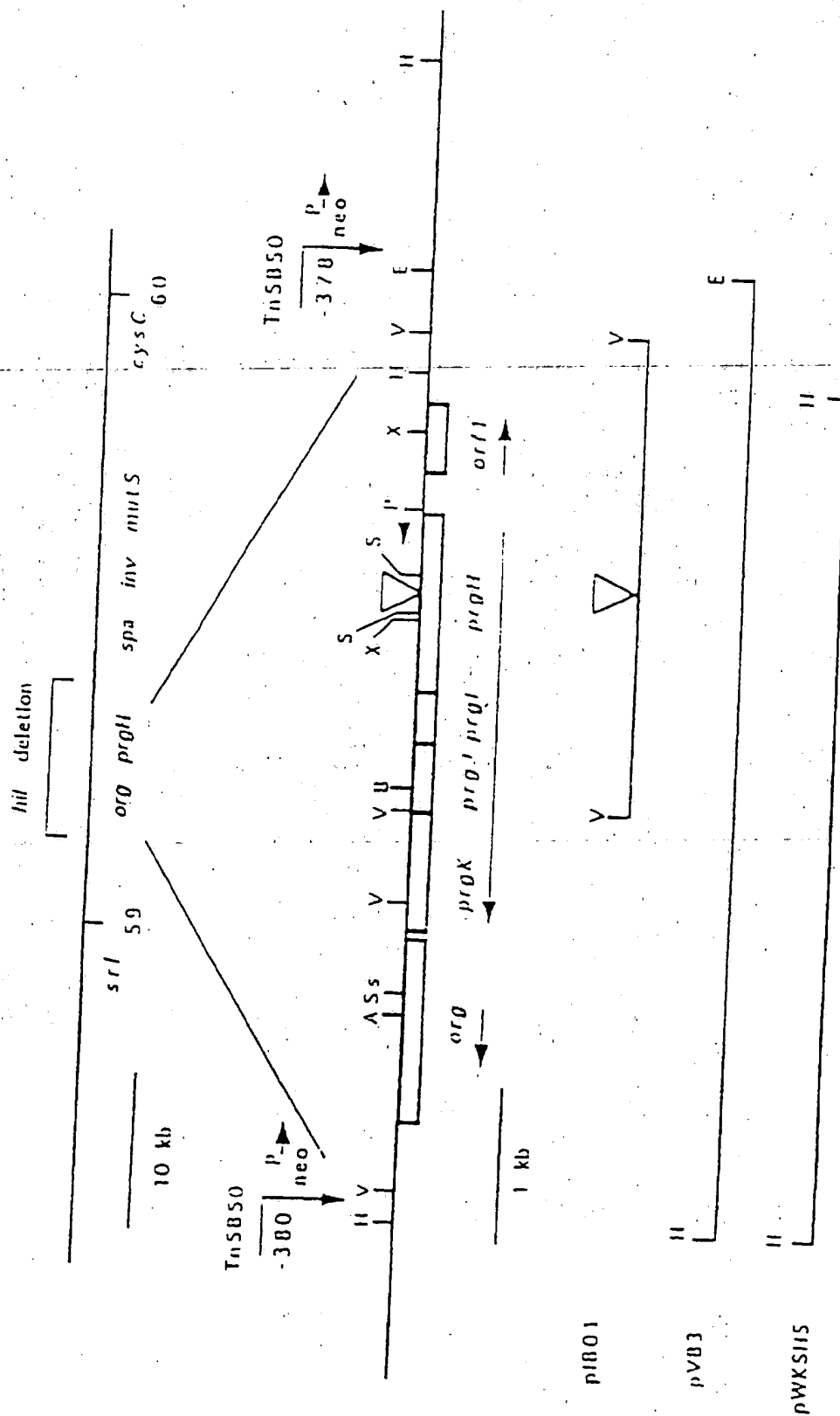
44. A substantially pure StpA polypeptide.

15 45. A method of dephosphorylating a protein, comprising contacting said protein with the polypeptide of claim 44 or an active fragment thereof.



1/27

Fig. 1



2/27

FIG. 2

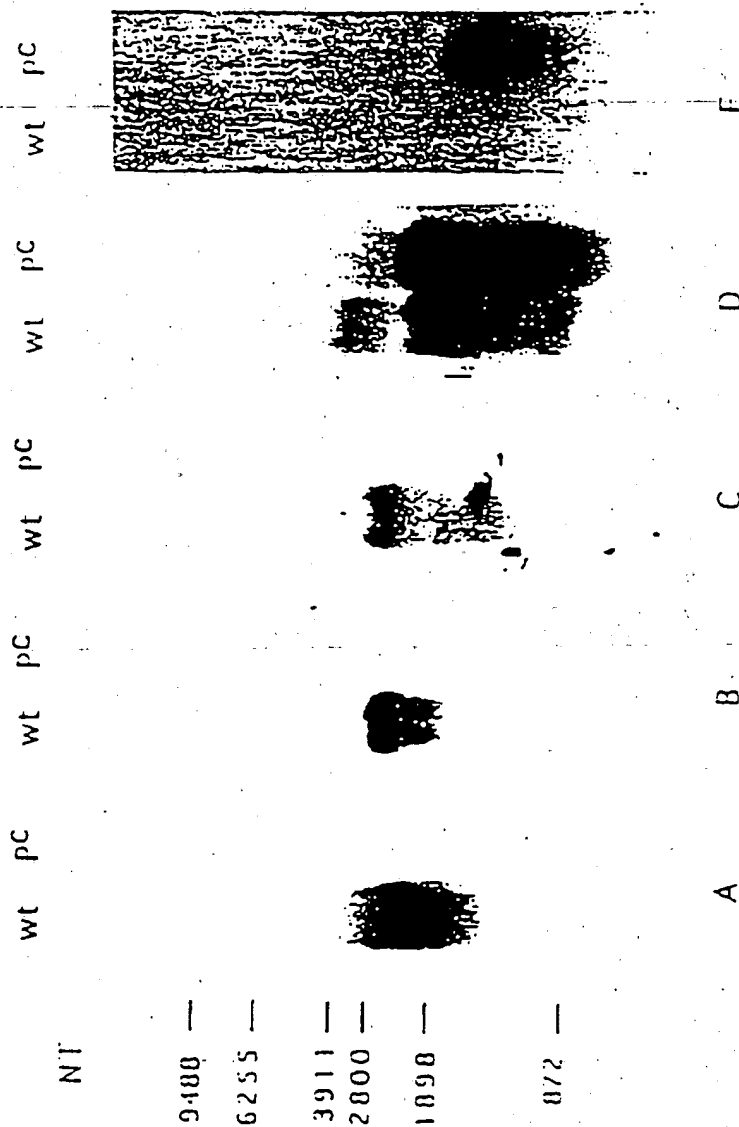


FIG. 3

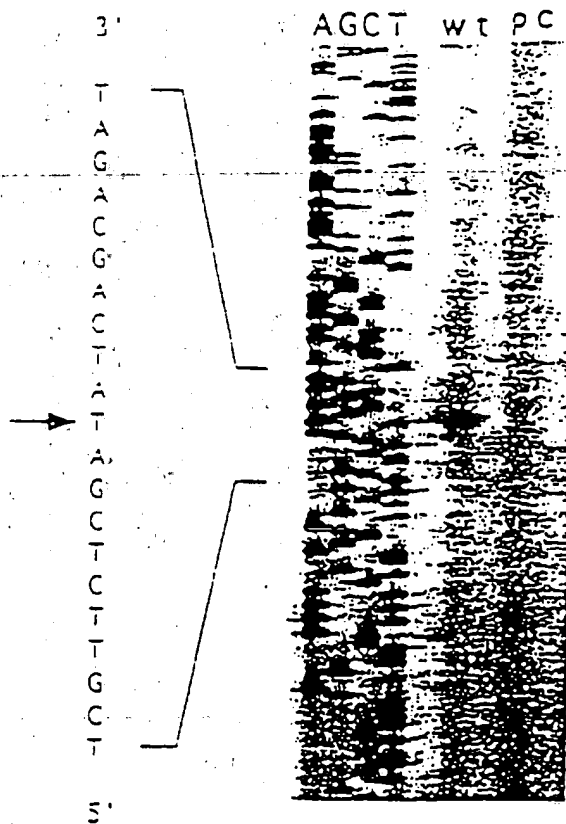


FIG. 4A

```

      * * * * *
PrqI 1  ...MATPWSGY.....LDDVSAKFTGTGVONLQTQVTEALDKLAAPSPDP 41
Mx1H 1  ...MSUTVPNDOWT.....LSSLSETFDGDTQTLOGETLALDKLAAPSPDP 44
YscF 1  ...MSNFSGFTKGNDAIDDAVAQTLLKPPADDANKAWIDSTAAIKOTPDNF 46

      * * * * *
PrqI 40  ALLAYQSKSLSEYNLYPNAQSNTVYKVPKDDAATIQNFR 80 (SEQ ID NO: )
Mx1H 46  QLLAEYQSKSLSEYTLYPNAQSNTVYKVPKDDAATIQNFR 80 (SEQ ID NO: )
YscF 49  ALLADQLQHSNKWSNTVYKVPKDDAATIQNFR 87 (SEQ ID NO: )

```

FIG. 4B

```

PrqI 1  MSIAITVPEANV...IGQAVNIRSMETDTVSLDDRLLQAFSGSAAATAVDKQT 50
Mx1H 1  ...PNVYEPVNVQVDFKASDPQSQET...ISSIEDVVSANYSDTKHDTQIQVSC 47

      * * * * *
PrqI 51  ITNRIEDPNLWTDPKELAISQEMISDYNLWVSMVSTLTKKGVGAVETLLRS 101 (SEQ ID NO: )
Mx1H 48  IMEMVSNPESLNPESLAAIQTTLSNYSIGVSLAGTLARNTVSAVETLLKS 97 (SEQ ID NO: )

```

FIG. 4C

```

PrqK 1  ...MIRPYLYTFLVMTLAGCKDK...DLKGLDQEQANEVIAVLQMHNTKANHI 49
Mx1H 1  ...MIRPYKGTFLFLMLIGCEQREELISNLSQEQANEVIAVLQMHNTKANHI 50
YscJ 1  ...MIRPYKGTFLFLMLIGCEQREELISNLSQEQANEVIAVLQMHNTKANHI 49

      * * * * *
PrqK 50  DSGKLGYSITVAEPDFTAAVYWKTYQLPPRPRVEIAQMFPADSLVSSPR 99
Mx1H 51  DGGKQGISVQVEKGTTFASAVDLPMYDLPNPERVDSIQMFPDLSLVSSPR 100
YscJ 50  FDKDGKELLLVEESOVAQAIDILKRGYPHESFSTLQDVFPKDGGLISSPI 99

      * * * * *
PrqK 100  AEKARLYSAIEQRLEQSLQTMGVLSARVHISYDIDAGENGRRPKPVHLS 149
Mx1H 101  AEKARLYSAIEQRLEQSLVSTIGGVISAKHVSYLE...EKNTISSKPMHIS 148
YscJ 100  EELARLYNKAQETISRTLSEIDGVLVARVHTVLPPEEQNNKGRKGVPAASAS 149

      * * * * *
PrqK 150  ALAVYERGSPLANQISCIKRFKNSFADVOYDNISVVL...SERSDAQL 195
Mx1H 149  VEAIVDSPKESELLVSNIRKFLQVTFSDVYENISVVL...TPHEHTVY 195
YscJ 150  VTEKHAADIQFOTVTPQIKQLVNNSTEGLAYDRISVILVPSVDVTRQSSH 199

      * * * * *
PrqK 196  Q...APGTPVKRNSFATSWIVLITLLSVMSAGFGVWYYKHYARNKKGITA 243
Mx1H 196  TVVQPVKEVKSEFLTNEVIVLFLGMVAVLITLLVWAFKTSWFKRNTKI 242 (SEQ ID NO: )
YscJ 200  P...RNTSILSIQVSEESKGRLLIGLLSILITLLPVTNLAQVFWLQRKK 244 (SEQ ID NO: )

PrqK 240  DDKAKSSNE 152 (SEQ ID NO: )

```

FIG. 5

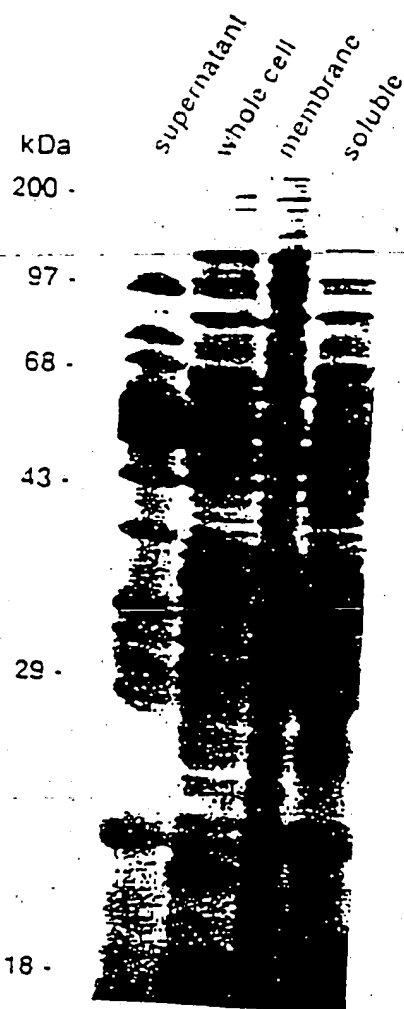
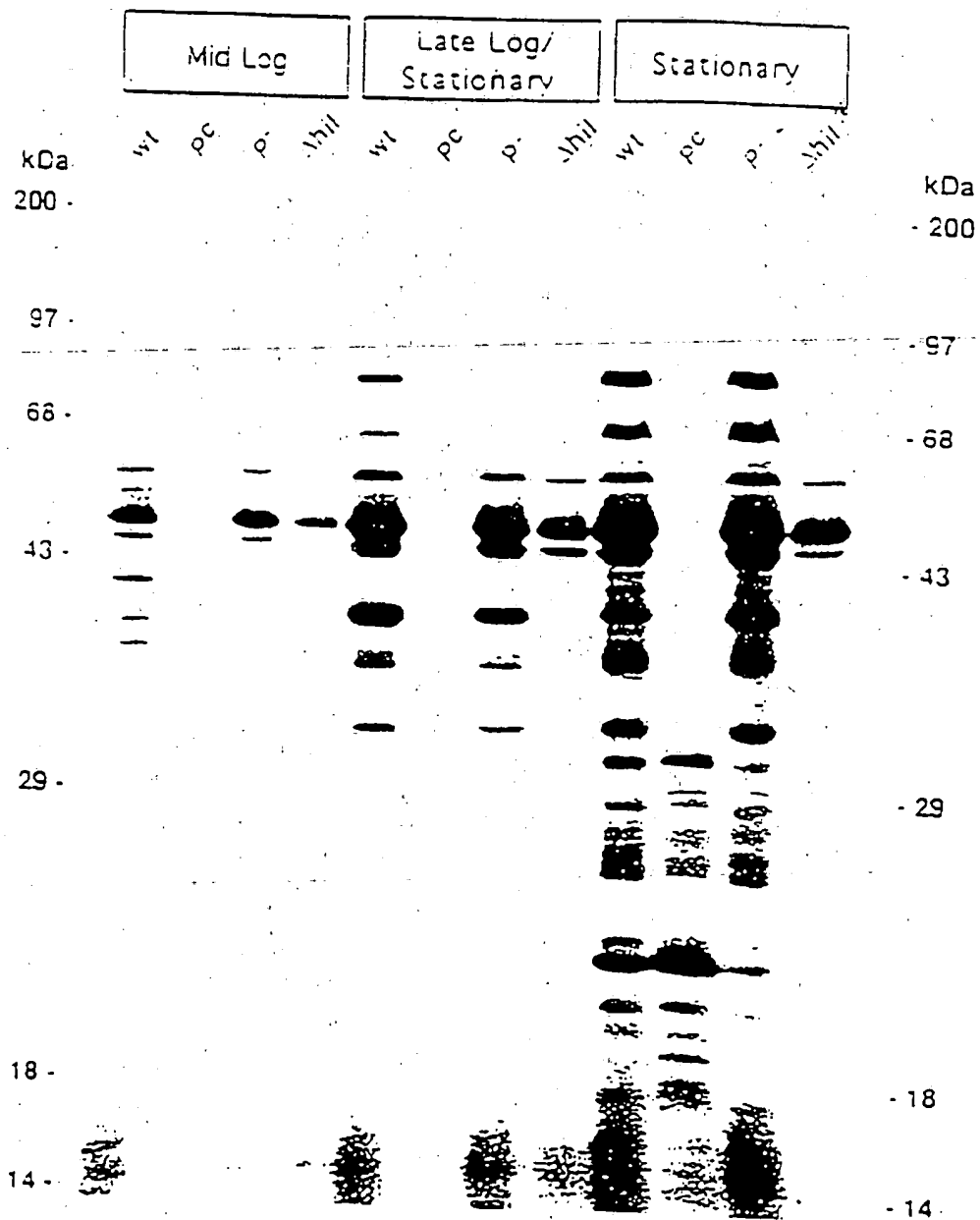


FIG. 6



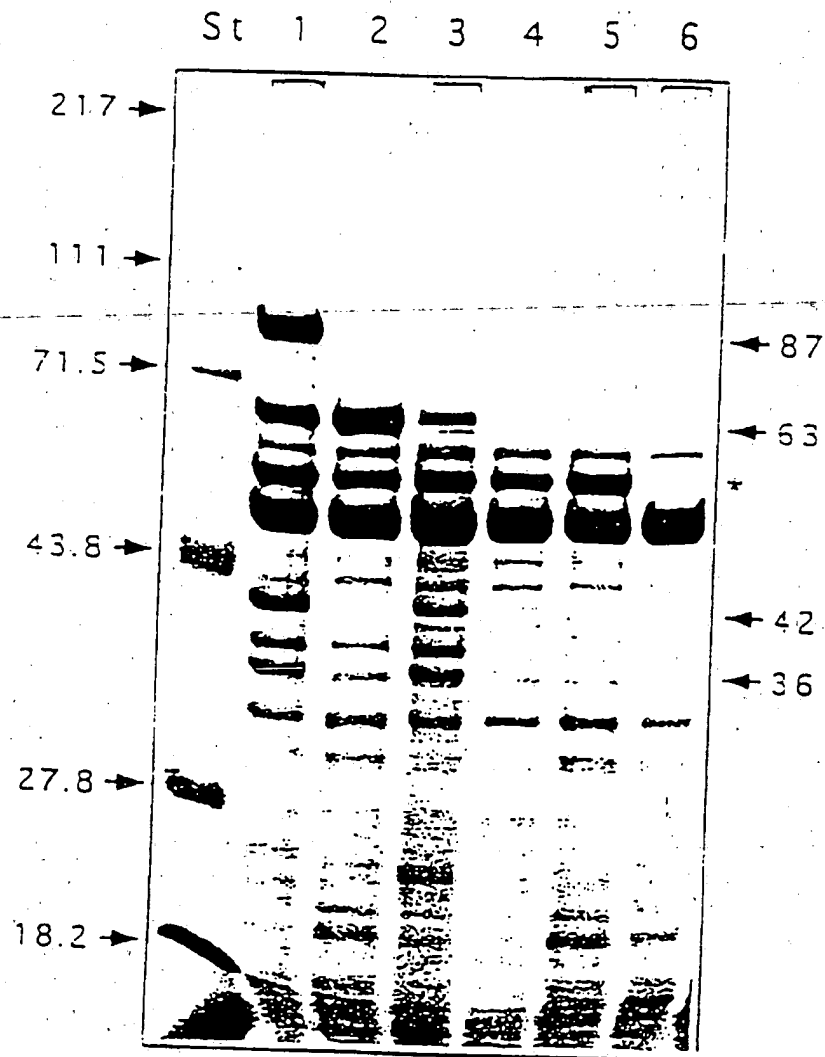
7/27

FIG. 7



8/27

FIG. 8





9/27

FIG. 9

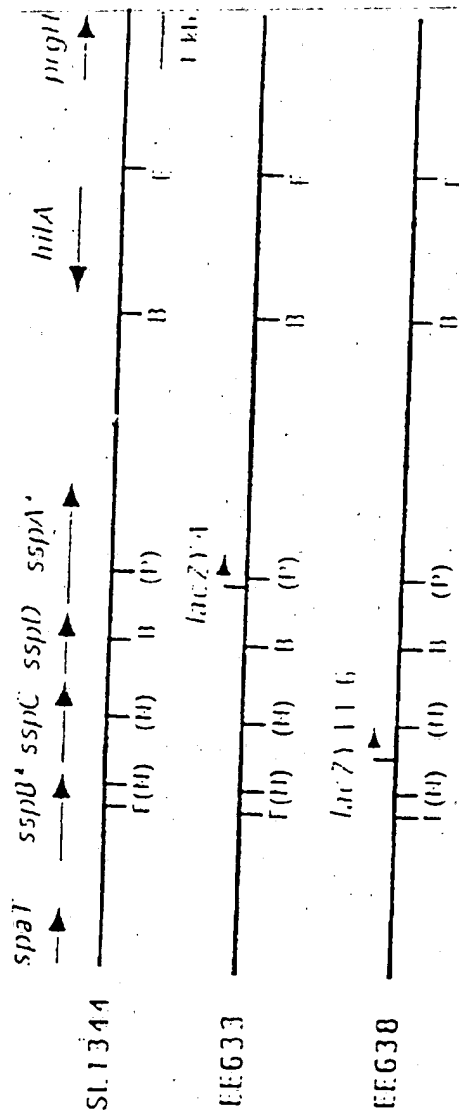
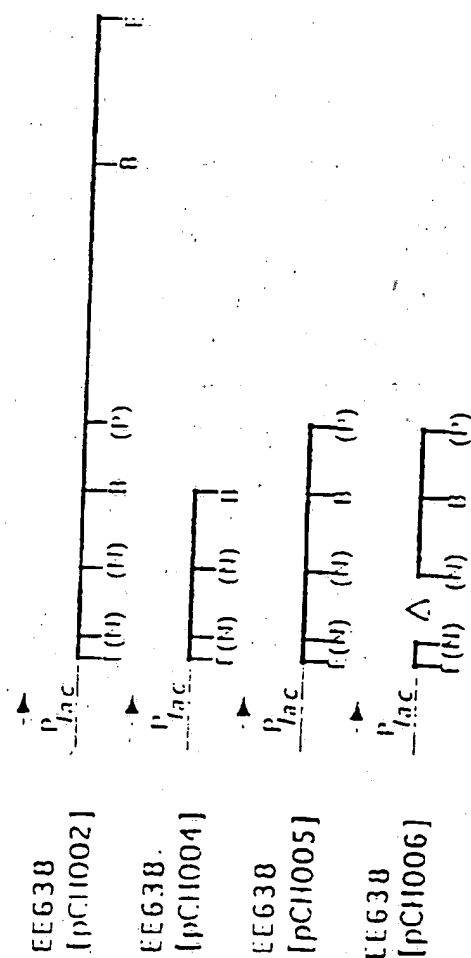


FIG. 10



invasion [% of inoculum]	Ssp		
	A	C	D
	(87)	(42)	(36)
17.5 ± 2.6	+	+	+
23.2 ± 3.5	-	+	+
0.1 ± 0.05	-	-	-

invasion [% of inoculum]	Ssp		
	A	C	D
	(87)	(42)	(36)
7.1 ± 2.3	+	+	+
0.2 ± 0.09	-	+	-
16.5 ± 2.7	-	+	+
0.1 ± 0.05	-	-	+

10/27

FIG. 11

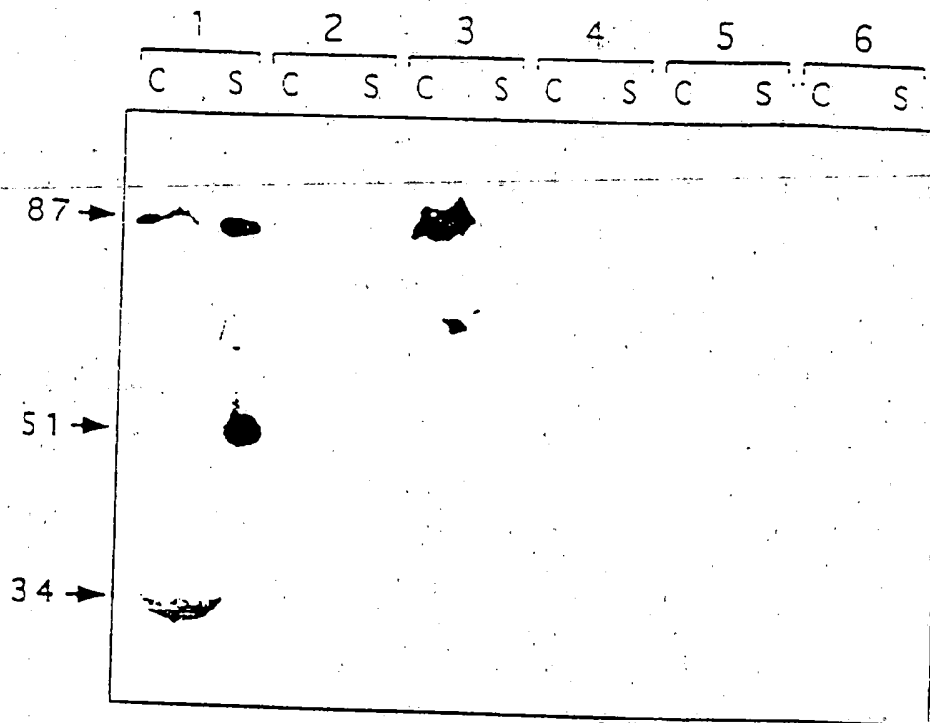


FIG. 12

5508     RKAEETNRINGCICXVLGALLTIVSVVAAVFTGASLALAAVGLAVHVADEIVKAATGVSTIQOALNPIMEHVLKPLMELIIEAITKALEGLVDRKQR  
 5508     RKAEEENRVHMGCVCKILGALLTIVSVVAAAFSGGASLALAAVGLAVHVADEIVKAATGVSTIQOALNPIMEHVLKPLMELIIEAITKALEGLVDRKQR  
 5508     MPAALLVRLSPLCHGDVIVVAVVCKGAAKLCALSKHNCETIKXLVPMVLYKOLONGSKLFTQGHORITSLGCVGSKHGLTNAISKELVGNTLHK  
 5508     MIGSILGATAGALVLAHVAVVCKGAAKLCALSKHNCETIKXLVPMVLYKOLONGSKLFTQGHORITSLGCVGSKHGLTNAISKELVGNTLHK  
 5508     VALCHEVTNTAQSAGGVAEQVETKNASEALADPHLRANDQIQMLKOSVEIEGENDKVTAELOKAMSSAVQONADASRFILROSRA (SEQ ID NO: 1)  
 5508     AVLLGESVNSATQAGGSVASAVFQNSASTHLAOLTLKYQVEDLSKYISEATEKFGQLOEVIADLLASHMSNOANRTDVAKALQQTITA 580 (SEQ ID NO: 2)

FIG. 13

550C     HLISNVGINPAATLNNHNSVENSSQASOSVSAKDILNSIGSSSKVSOLGVESYTEPACAGVLTOTPGTITSPFKASIONTCHMODLHALLA  
 550C     HLOKQFCNKLLDTNKENVMEION...TKPTQTLVTDISTKQTSSETOKSONYQOIAHPLVCKHNPVLTTLNDCCQLKLSQVCHDSEIDARLT  
 550C     NMVTTKANENVQTLREQAEVCKFFDISCHSSSAVALAAANTLMLTLNOADSKLSCKLSLVSFDAKTTASSHMRCHCITVCSITPEPLLOLQITGVG  
 550C     DKMKDLSEMSHTLTPEPT...LDISSLSNAVSLIISVAVLLSALRTAETKLSQSLSLIAFDATKSAAEIVRQGLAALSSTITGAVTQVGTGCG  
 550C     AKLEYKGLQNERCPKHNACKIDKLTTESHSIKNVLNGONSIKLCAGVDSLSLNMHKTGTDAKTNLNOATLKSNACTSAATESLCKIKDSNKQISPEHOA  
 550C     AKKTHSGISDQKGLRKNLATAOSLEKELAGSKLGLNKQIDTNTSPQTHS...STKFLGKNKLAPD...NISLSTEHKT  
 550C     ILSKRLESVESDIRLEONTMCHTRIDARKMCHTCDLHKNSVTVGCIAGASGOYAATOERSEQQISQVMHRVASTASOEARESSRKSTSLIQEHLKTHES  
 550C     SLSSPOISLQDKIDTQRRTYELNTLSAQQKQNHGRATHETSAVAGNISTSGGRYASALEEEELISQASSKQAEASQVSKEASOATHQLKLNHIDS  
 550C     INQSKASALARIAGNIRA 409 (SEQ ID NO: 3)  
 550C     INQSKNSAASQIAGNIRA 332 (SEQ ID NO: 4)

FIG. 14

550D     MLNIQNYASAPHPGIVAERPQTSASEHVEIVVPSITTEHRTGDIISLQAATKIHQAQTLQINATISEENNDERSKGO...LCTSS...LN  
 550D     MHITTLTNSISTSSFSNNTHCSSTETVNSDIKTTTSSHVPYSSLTHLNOTLHNIRTTNOALKKE...LCKTLTKTSLIEELHSSQISHOWNKSAOLLO  
 550D     ALAKSGVSLSAEQNENLRSAPTSALFSASPMAOPRTTISDAEIMOMVSONISAIGOSYLGVFMKMMVAVYTFDYQAFSDILSKHGMLLPCKDQNT  
 550D     ILSRNEYPINKDARELLH...SAPKEAELDQGM...ISHRELNAKIANINDINEQYLKYY...EHAUSSITCMYODFSAVLSSLACNISPGGNDGNS  
 550D     VKLOVTSKLNOLNSLVNKHNOINSNTFYDAVSKRTARRRRECHLSENLPHSCLKSYGSGVAVTDLTPLOKMWCDIGGLSAPGKDSKLEHONAKYQANC  
 550D     VKLOVNSLKKALEELKEKXCKP...LYPANNTVSOEQANKLTELCTICKVSKNGGCVSINHTPEDHMLKSLDHLGCHG...EVLONAKYQAWN  
 550D     SGFKAOEENMKTTLOTLTCKYSNANSLYONLVKLSSTESTSLEPPKPSCKD 333 (SEQ ID NO: 5)  
 550D     AGFSAEDETMMKNLQTLVOKYSNANSIFONLVKLSSTESTSCTDOKLFLHF 332 (SEQ ID NO: 6)

FIG. 15

550A     MVTSVRTQPPVIMPQSOTEIKTRPRIC 332 (SEQ ID NO: 7)

FIG. 16

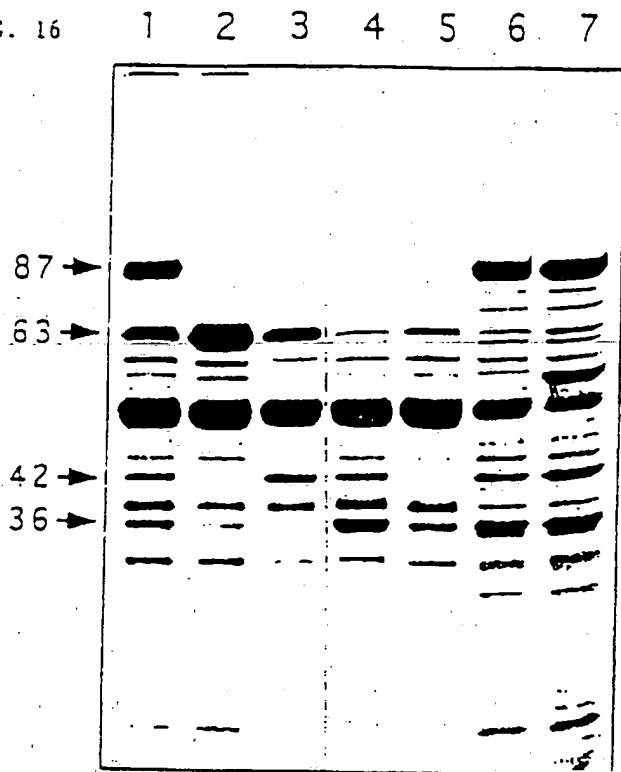


FIG. 17

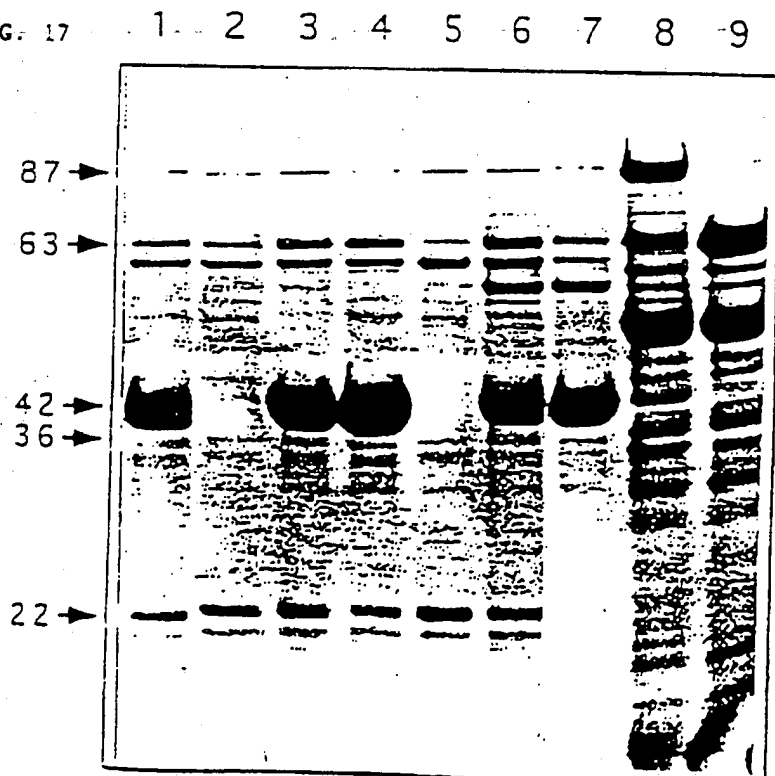
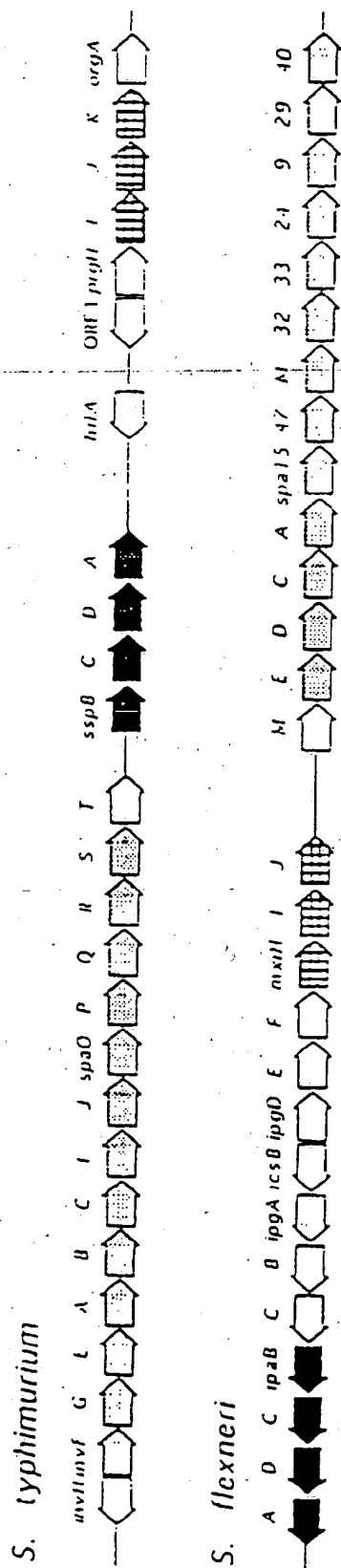


FIG. 1B



14/87

CCGAAAGCCGAGGAAACGAAACCGCATTATGGGATGTATCGGGAAAGTCCTC  
CGCCCGCTGCTAACCATTGTCAGCGTTGTGGCCCGCTGTTTTAACGGTGGGGCG  
AGTCTGGCGCTGGCTGGGGTGGGAGTGGGGTAAATGGTGGCCGATGAAATTG  
GAAGGCCGCGACGGGAGTGTCTTTATTCAGCAGCCGCTAAACCCGATTATG  
GAGCATGTGCTGAAGCCGTTAATGGAGCTGATTGGCAAGGCCGATTACCAAA  
CGCTGGAAGGATTAGGCGTCCATAAGAAAACGGCAGAGATGGCCGGCAGC  
ATTGTGGTGGGATTGTCCCGCTATTGCCATGGTGGCGGTCATTGTGGTGGTC  
CCAGTTGTCCGGAAGAGCGCGCGCGCGGAAACTGGGTAAACCGCTGAGCAAA  
ATGATGGCGGAAACGATTAAAGAAATTGGTGCCTAACGTGCTGAAACAGTT  
CGCGCAAAAACGGCAGCAAACTCTTTAACCAGGGGATGCCAACGTATTACTAG  
CGGTCTGGGTAAATGTGGGTAGCAAGATGGCCCTGCCAAGCAATGCCTTAAG  
TAAAGAGCTGGTAGGTAATAACCTAAATAAAGTGGCGTTGGGCATGGAAG  
TCACGAATACCGCAGCCAGTCAGCCGGTGGTGTGGCCAGGGCGTATTATTT  
AAAAATCCAGCGAGGCGCTTGCTGATTTTATCTTCCGCGTTTTGCCATGG  
ATCAGATTACGCAGTGGCTTAAACAATCCGTAGAAATATTTGGTGAAAAC  
CAGAAAGGTAACGGCGGAACTGCAAAAAGCCATGTCTTCTGCGGTACAGCAA  
AATGCCGATGCTTCGCGTTTTATCTTCTTCCAGAGTCGCGCATAA

FIG. 19 (SEQ ID NO:1)

15/87

ATGTTAATTAGTAAATGTGGGAATAAAT  
CCCCCGCGTTAATTAATAATCATTCGTGAGAATAGTTCCACAGATAGCT  
TCCGAATCCGTTAGCGCTAAAGATATTCTGAATAGTATTGGTATTACGAGC  
AGTAAAGTCAGTGACCTGGCGTTGAGTCTACACTGACCGCGCTGCGGCAGG  
GGTATTAAACGCAAACCCCGGAACGATCACGTCCTTTTTAAAGCCAGTAT  
TCAAATAACCGACATGAATCAGGATTTGAATGCTCTGGCAAAATAATGTCA  
CGACTAAAGCGAATGAGCTTGTCCAAACCCAGTTACCGGAGCAGCAGGCAG  
AACTCCGAAAGTTTTTGATATTACCGGAATGTCTCCAGTCCCGTTGCGCT  
GTTGGCTGCGCGGAATACGTTAATCTGACGTTGAACACAGGCTGATAGCAA  
ACTGTCTGGTAAGTTGTCAITAGTCAGTTTTGATGCAGCTAAAACGACGGC  
AACTTCATGATCCCGGAAGGGATGAATGCGTTGTTCGGTAGTATTCCCAAG  
ACCGCGCTTCAGTTGGCGATCACTGGCGTGGCGCCAAACTGGAATATAAGG  
GCTGCAGAATGAAAGAGGGCGGCTTAAACATAATGCCCGCGAAGATCGAT  
AAACTGACCACTGAAAGCCACAGTATTAAAAACGTTGCTGAACGGGCAGAA  
TAGCGTCAAACTCGGTGCTGAAGGCGTTCGATTCTCTGAAATCGTTAAATAT  
GAAGAAAACCGGTACCGATCCGACGAAAAATCTTAATGATCCGACCGTTA  
AATCTAATGCCCGAACCAGCGCCACCGAAAGTCTGGGTATTAAAGACAGT  
AATAAACAAATCTTCUCCCTGAACATCAGGCTATTCTGTTCGAAACGTCTTGAG  
TCGTTCGAATCCGATATTCTGTTGAGCAGAAATACCATGGATATGACCCGA  
ATCGATGCGCGCAAGATGCAGATGACGGGCGATCTGATTATGAAGAACTCG  
GTCAAGGTCCGTTCTATTGACGGGGCTTCUCCCGAGTACCGCGCTACTCAGGA  
ACGTTCCGAGCAGCAAAATTAGCCACCTTGAATAACCGGGCTTCCAGCAACCA  
TCGGACGAAGCCCGTGAAAGTTACAGTAAATCGACCAGCCTGATTACAGGAA  
ATGCTGAAAACAATGGAGAGCATTAAACCAGTCCGAAACCATCCCCACTCGCT  
CKTATCCGAGGCAATATTCCCKTTAA

ATGCTT

AAATATTC~~AAAA~~ATTATTC~~CGC~~TTCTCCTCATCCGGGGATCGTTT~~CCCC~~GAACGGCC  
GCAGAC~~TTXX~~TTGGCGAGCGAGCACCTCTAGACTGCGGTGGTACCGTCTACCAC  
AGAACATCGCGGTACAGATATCATTTCATTATCGCAGGCGCGCTACTAAAAAT  
CCAOCACGGACAGCAGACGCTGCAGTCAACGCCACCGATCTCTGAAAGAGAAT  
AATGACGAGCGGCACCGCTGGCGCGCCAGCAGTTGACCAGCAGCGCTGAATGGCGT  
GGCGAAGTCCCGCGGTGTCTATTATCCGCAGAACAAAATGAGAACCTTGGCGAG  
CGCGTTTTCTGCGGCGACGTGGCGCTTATTTAGCGCTTGGCTATTC~~CCCC~~CAGCGG  
AGAACAAACCATTTCTGATGCTGAGATTTGGGATATGGTTTCCCAA~~AA~~ATAT  
ATCGGCGATAGGTGACAGCTATCTGGCGGTTTATGAAAACGTTGTGCGCAGTC  
TATACCGATTTTTATCAGGCGCTTCACTGATATTCTTTCCAAAATCGGAGGCT  
GGTTATTTACCAGGTAAGGACCGTAATACCGTTAAGCTAGATGTTACCTCAC  
TCAAAAATTGATTTAAACAGTTTAGTCAATAAATATAATC~~AA~~ATAAACA  
GTAAATACCGTTTTATTTCCAGCGCAGTCAGGCAGCGCGGTTTAAAGTAGCCAC  
TGAAGCGGAAGCGAGACAGTGGCTCAGTGAATTGAATTTACCGAATAGCTG  
CTGAAATCTTATGGATCCGGTTATGTCGTCACCGTTGATCTGACGCCATTAC  
AAAAAATGGTTCAGGATAATTGATGGTTTAGGCGCGCGGGGAAAAGACTCA  
AAACTCGA~~AA~~ATGGATAACGCCAAATATCAAGCTGGCAGTCGGGTTTTAA  
AGCGKAGGAAGAAAATATGAAAACCACATTACAGACGCTGACGCA~~AAA~~  
TATAGCAATGCCAATTCAATTGTACGACAACCTGGTAAAAGTGCTGAGCAGT  
ACGATAAGTAGCAGCCTCGIAAACCGCCAAAACCTTCTGCAAGGATAA

FIG. 21 (SEQ ID NO:3)



ATGGTTACAAGTGTAAGGACTCAGCCCCCGTCA  
TAAATGCCAGGTATGCAGACCGAGATCAAAACCCAGGCCACGAATCTTCCGG  
CGAATCTTCCGCAGTCAGAGAAAGTGCCACACCGACGCTGTCAGGGGAAA  
TTAAAGGCCCGCAACTGGAAGATTTTCCCGCGCTGATCAAACAGCGGAGTCT  
CGATGC

(SEQ ID NO:4)

RKAEETNRIMGCIGKVLGALLTIVSVVAAVFTGGASLALAAVGLAVMVA  
DHIVKAATGVFSFIQQALNPIMEHVLKPLMELIGKAITKALEGLGVDRKRQR  
WPAALLVRLSPLCHGDAVIVVVAVVGKGAAAKI.GNALSKMMGETIKKL  
PNVLKQLAONGSKLFTQGMQRITSGLGNVGSKMGLQTNALSKIELVGNTLNK  
VALGMEVTNTAAQSAGGVAEVGFKNASEALADFMLARFAMDQIQQWLK  
QSVEIFGENQKVTAELQKAMSSAVQQNADASRFILRQSRAZ

(SEQ ID NO:5)

FIG. 22

MLISNVGINPAAI.NNHSVENSSQTASQSVSAKDII.NSIGISSSKVSDLGI.SP  
 TLSAPAPGVLTQTPGTITSFLKASIQNTDMNQDI.NALANNVTTKANEVVQT  
 QLREQQAEVKGFFDISGMSSSAVALLAAANTLMLTLNQADSKLSGKLSLVSF  
 DAAKTTASSMMREGIMNALSISISQSALQI.GITGVGAKLEYKGLQNERGALK  
 HNAAKIDKI.TTESHSIKNVLNGQNSVKLGAEGVDSLKSLNMKKTGTDATKNL  
 NIDATLKSNAAGTSATESLGIKDSNKQISPEHQAILSKRI.ESVESDIRLEQNTMD  
 MTRIDARKMQMTGDLIMKNSVTVGGIAGASGQYAA.TQERSIQQISQVNNRV  
 ASTASIDEARESSRKSTSIHQEMLKTMESINQSKASAL.AAIAGNIRAZ

(SEQ ID NO:6)

MLNIQNYASAPHPGIVAERPQTPSASEHVETA.VVPSTTEIIRCTDIISLSQAA  
 TKIHQAQQTLQSTPPISEENNDERTLARQQLTSSI.NAI.AKSGVSLSAEQNENI.  
 RSAFS.APTSALFSASPMAQPRTTISDAEIWDMVSNISAIGDSYLGVEYENVV  
 AVYTDFYQAFSDILSKMGGWLLPGKDGNTVKLDVTSCLKNDLNSLVNKYNQI  
 NSNTVLTPAQSGSGVKVATEAEARQWLSELNLPNSCLKSYGSGYVVTVDLT  
 PIQKMVQDIDGLGAPGKDSKILEMDNAKYQAWQSGFKAQEENMKITLQTLTQ  
 KYSNANSLYDNLVKVLSSTISSI.ETAKSIFLQGZ

(SEQ ID NO:7)

MVTSVRTQPPVIMPGMQTEIKTQATNLAA.NLSAVRESATATLSGEIKGPQL  
 EIDFPALIKQASLD

(SEQ ID NO:8)

ATGCATTATTTTTTATCATCGTAATCTGGTTCCTTAGCATAAATA  
CGGCATGGGCTGATTCTGGCTTCAGGCTGAAAAAATGTTCAATAATTGAATC  
CGAACTACTTTACGCTATCGCCCAGCAGGAATCCGGGATGAAACCTGCKKKC  
ATTGGTCATAACCGAGATGGTTC AACCGATCTTGGCCTGATGCAAATTAAC  
AGCTTCCATATGAAAAGGCTGAAAAAATGGGGATTAGTGAAAAACAGT  
TGTTACAGGACCCCTGCATTTCCTGTCATTGTGGCGGACCTCCATTTTATCAGA  
TATGATGAAAATCTACGGTTATAGCTGGGAGGCGGTTGGCGCTTATAATCC  
CGGGACGTCCCGGAAACGATCCGATATTAAGGAAACGTTATGCTAAAAAAA  
TTTGGGAGAATTACAGAAAATTAAAAGGAATGTCAGCAGAAAGAGAAAAA  
ACAAAAGACTTCTATCGCGGCAAAACAAATAA

(SEQ ID NO:9)

ATCAGCTTGGCGTGGTCATAAGCAACTGGGCTTGCATTGCTTTAGTTGTAC  
AAACTGTGCAGGCGTCTCCAGCATTTCTATTGTTCCGTGAATCCCGAATCT  
GCACGTACCTGCTCCAGATTACTATGAGGATTATCCTTAAGTACAAGGGCCG  
CCGCCATCGTCCGGTCTTCCCACTCCGCCAGACAATGAATCATCGGTAAB  
TGCTTATCTGATGAACTACCKKXXGGCCCGGCATTTTGGTTACTATTTTCAC  
CCTATCCGCCAGGTATTCTAACTGATCCGTAGACGGTAAACGGCTGGTGATCTG  
GCCAATTTTCACATGCAATACCGGGATTGTATACCGCTTTCCCGCAGGAC  
AGTTGCATATTGTATTGGTCTATCGCTTCTCCCTGACCTGGCTGAGCTCTTTT  
TTGGCTGTGGTATGCAOCTCCGCCAAAGGTCTAGCTCCCTCTGAAATAGCTTGG  
TAATTGTTTTGXXKKAATCTGATCTCCGACGTTAACACCACCAGGCACGAGC  
ATTCTTTTTCAAGAAGCATTTTCATATCGGCTTCCAGCGCATCCCGGCCGATT

(SEQ ID NO:10)

20/27

MHYFFHVIWLLSINTAWADSGFRLKKCSILNPNYFTLSPSRNRRZNLAPLVI  
TEMVQPILAZCKLTASLZKGZKKWGLVKNSCYRTPAFLSLWATSILSDMMKI  
YGYSWEAVGAYNAGTSPKRSDIRKRYAKKIWENYRKILKGMSAEKKNKRLSIA  
ANK

(SEQ ID NO:11)

WPGTICGQQHSINQQTQVKLSDGMPVPVIRLTFDGKPVALAGIRTQKIRPDR  
LEAHMKMLLEKECSCLVVLTSERSDAGKTITTYIRGSYTI'GLVHTNSQKVSSA  
SQGEAIDQYNMQLSCGEKRYTIPVLHVKNWPDHQPLPSTDQLEYLADRVKNS  
NQNGAPGRSSDKILPMIICLGGVGRGTGTMAAALVLKDNPHSNLEQVRADF  
RIHGTIECWKTPAQFVQLKAMQAQLLMTTAS

(SEQ ID NO:12)

ATGCGTGATTGCCTGAATAAACGGCAATCCAGTGCTTAACGTG  
GGAGCGTCAGGTCTTACCACCTTACCAGACCGTTTACCACCGCATAT  
TACAACACTGGTTATTCTCTGATAATAATCTGACCAGCCTGCCGGAG  
TTGCCGGAAGGACTACGGGAGCTGGAGGTCTCTGGTAACCTACAAC  
TGACCAGCCTGCCATCGCTGCCGCAGGGACTACAGAAGCTGTGGGCC  
TATAATAATTGGCTGGCCAGCCTGCCGACGTTGCCGCCAGGACTAGG  
GGATCTGGCGGTCTCTAATAACCAGCTGACCAGCCTGCCGGAGATGC  
CGCCAGCACTACGGGAGCTGAGGGTCTCTGGTAACAACCTGACCAGC  
TGCCGCGGCTGCCGTGAGGACTACAGAAGCTGTGGGCCCTATAATAA  
TCCGCTGACCAGCCTGCCGGAGATGTGCCAGGACTACAGGAGCTGG  
ATGCTCTCTATAACCAGCTGACCCGCCCTGCCGCAAAGCCTCACGGGT  
CTGTCTTCAGCGGCACGCGTATATCTGGACGGGAATCCA CTGTCTGT  
ACGCACTCGTGACAGGCTCTGCCGACATCATTTGGCCATTACAGGCATC  
AGGATACACTTCGATAATGGCGGGGCCCTTCCGTCCCCGGGAAGCCCCG  
GCACTGCACCTGGCGGTCCGTGACTGGCTGACCTCTGCACGGGAGGGG  
GAAAGCGGCCCAAGGCAGACAGATGGCAGGCGTTCCGGA CTGGAAGATA  
ACGCCGCCGCCCTTCAGCCTGGTCC TGGACAGACTGCCGTGAGACGGAA  
AACTTCAAAAAAGACGCGGGCTTTAAGGCACAGATATCATCCTGG  
CTGACACAACTGGCTGAAGATGCTGCCGTGAGAGCAAAAACT  
TTGCCATGGCAACAGAGGCAACATCAACCTGCCGAGGACCGGGTCA C  
ACATGCCCTGCACCAGATGAATAACGTACAACTGGTACATAATGC  
AGAAAAAGGGGAATACGACAACAATCTCCAGGGGGCTGGTTTTCCAC  
GGGGCGTGAGATGTTCCGCCCTGGCAACACTGGAACAGATTGCCCGGG  
AAAAAGCCGGAACA CTGGCTTTAGTCGATGACGTTGAGGTCTATCT  
GGCGTTCCAGAAATAAGCTGAAGGAATCACTTCAGCTGACCAGCGTG  
ACGTCAGAAATGCCCTTTCTTTGACGTTTCCGGCGTGACGGTTTCA  
GACCTTCAGGCTGCCGACGTT CAGGTGAAAAACCGCTGAAAAACAGCG  
GGTTCAGTAAATGGATACTGCAGTGGGGGGCCGTTACACAGCGTGCT  
GGAACGCCAAAGTGCCGGAACGCTTTAACGCGCTTTCGTGAAAAGCA  
AATATCGGATTATGAAGACACGTACCGGAAGCTGTATGACGAAGT  
GCTGAAATCGTCCGGGGCTGGTCGACGATAACCGATGCAGAACGTACT  
ATCGGAGTAAGTGCGATGGATAGTGCGAAAAAAGAA TTTCTGGA  
TGCCCTGCCGCGCTTTGTGGATGAGGTGCTGGGTAGCTATCTGACAG  
CCCGGTGGCGTCTTA ACTGA

22/27

MRIDCLNNGNPVLNVGASGLTTLPDRLPPHITTLVIPDNNLTSLPELPEGLRE  
LEVSGNLQLTSLPSLPQGI.QKLWAYNNWLASLPTLPPGLGDLAVSNNQ LTS  
I.PIEMPPAI:RELKVSGNNLTSLRALPSGLQKLWAYNNRLTSLPEMSPGLQELD  
VSHNQLTRLPQSLTGLSSAARVYI.DGNPLSVRTIDRLCGIIWPFRHQDTLRY  
GGAFKPREARAI.HI.AVAIDWLTSAREGEAAQADRWQAFGLDNAAAFSLV  
LDRLRETENFKKDAGFKAQISSWLTQLAEDAAI.RAKTFAMATEATSTCEDRV  
THALHQMNNVQI.VHNAEKGEYDNNLQGLVSTGREMIFRLATLEQIAREKAGT  
LALVDDVEVYLAFQNKLELTSVTSEMRFFDVSGVTVSDLQAADVQVK  
TAENSGFSKWII.QWGPLHSVLERKVPERFNALREKQISDYEDTYRKLYDEVLK  
SSGLVDDTDAERTIGVSAMDSAKKEFLDGLRALVDEVLGSYLTARWRLNZ

FIG. 27 (SEQ ID NO:14)

23/27

CCCAAAGCCGAGGAAACGAACCCATTATGGGATGTATCGGGAAAGTCCTC  
GGCCCGCTGCTAAOCCATTGTACGCTTGTGGCCGCTGTTTTAOCGGTGGGGCG  
AGTCTGGCCCTGCCITGCCGTGGGACTTGGCGTAAATGGTGGCCGATGAAATTTG  
GAAGGCCGGGACCGGAGTGTCTTTATTTCAGCAGGCGCTAAACCCGATTATG  
GAGCATGTGCTGAAGCCGTTAATGGAGCTGATTGGCAAGGCGATTACCAAA  
CCGCTGGAAGGATTAGGCGTCGATAAGAAACCGCCAGAGATGGCCGGCAGC  
ATTGTTGGTGGGATTGTCCCGCTATTGCCATGGTGGCGGTCAATTGTGGTGGTC  
GCAGTTGTGGGAAAGGCCGGCGCCCGAAACTGGGTAACGCGCTGAGCAAA  
ATGATGGGCGAAACGATTAAAGAAAGTTGGTGCCTAACGTGCTGAAACAGTT  
GGCGCAAAACGGCAGCAAACTCTTTACCCAGGGGATGCAACGTATTACTAG  
CGGTCTGGGTAATGTGGGTAGCAAGATGGCCCTGCCAAACGAATGCCCTTAAG  
TAAAGAGCTGGTAGGTAATACCCCTAAATAAAGTGGCGTTGGGCATGGAAG  
TCACGAATACCGCAGCCCAGTCAGCCGGTGGTGTGGCCGAGGGCGTATTATTT  
AAAAATGCCAGCGAGGCGCTTGCTGATTTTATGCTCCGCCCTTTGCCATGG  
ATCAGATTTCAGCAGTGGCTTAAACAATCCGTAGAAATATTTGGTGAAAAC  
CAGAAAGCTAACGGCGGAAGTGCAAAAAGCCATGTCTTCTGCGGTACAGCAA  
AATGCGGATGCTTCGCGTTTTATTCTGCGCCAGAGTCGCGCATAAAAACTGC  
CAAAAATAAAGGGAGAAAAATAATGTTAATTACTAATGTGGGAATAAAAT

(SEQ ID NO: 15)

FIG. 28 (1 of 3)

CCCGCCGCTTATTAAATAAATCATTCTGTTGAGAATAGTTACACAGACAGCT  
TCCGAATCCGTTAGCGCTAAAGATATTCTGAATAGTATTGGTATTAGCAGC  
AGTAAAGTCAGTGACCTGGGGTTGAGTCTACACTGACCGGCKCTGCCCCAGG  
GGTATTAAACGCCAAACCCCGGAACGATCACGTCTTTTAAAAAGCCAGTAT  
TCAAATAACCGACATGAATCAGGATTTGAATGCTCTGGCAAAATAATGTCA  
CGACTAAAGCGAATGAGGTGTGCAAAACCCAGTTACGCCAGCAGCAGGCAG  
AAGTCCGGAAGTTTTTGATATTAGCGGAATGTCTTCCAGTGCCGTTGCGCT  
GTTGGCTGCGCGGAATACGTTAATGCTGACGTTGAACCAAGGCTGATAGCAA  
ACTGTCTGGTAAAGTTGTCAATTAGTCAGTTTTGATGCAGCTAAAACGACGGC  
AACTTCATGATGCGCGAAGGGATGAATGCGTTGTCCGGTACTATTTCCAG  
ACKCGGCTTCAGTTGGGGATCACTGGCGTGGGCGCCAACTGGAATATAAGC  
GGCTGCAGAATGAAAGAGCGCGCCTTAAACATAATGCCCGGAAGATCGAT  
AAACTGACCACTGAAAGCCACAGTATTAAAAACCTGCTGAACGGGCAGAA  
TAGCGTCAAACTCGGTGCTGAAGGCGTGGATTCTCTGAAATCGTTAAATAT  
GAAGAAAACCGGTACCGATCCGACGAAAAATCTTAATGATGCGACCGCTTA  
AATCTAATGCCGGAACCAAGCGCACCGGAAAGTCTGGGTATTAAAGACAGT  
AATAAACAAATCTTCCCTGAACATCAGGCTATTCTGTGGAACCGTCTTGAG  
TCTGTGGAATCCGATATTCTGTCTGAGCAGAAATACCATGGATATGACCCGA  
ATCGATGCGCGCAAGATGCAGATGACCGGCGATCTGATTATGAAGAACTCG  
GTCACGGTCCGTTTATTGACGGGCGCTCCGGCAGTACGCGCTACTCAGGA  
ACGTTCCGAGCAGCAAAATTAGCCAGTTGAATAACCGGGTTGCCAGCACCGCA  
TCGGACGAAGCCCGTGAAAGTTACAGTAAATCGACCAGCTGTATTACAGGAA  
ATCTTGAAAAACAATGGAGAGCATTAACCAGTCGAAAGCATCCGCACTCGCT  
GCTATCCGAGGCAATATTCCGCTTAATCTGAAAGGTCACTATACGCCATC  
ATGGGTGTGATTTAATCGCGCTCTGATGGCGAACTGGGGATATTATGCTT  
AATATTCAAAATTAATCCGCTTCTCTCATCCGGGGATCGTTGCGGAACGGCC  
GCAGACCTTCTCGCGAGCGAGCACTTGGAGACTGCGGTGGTACCGTCTACCAC



AGAACATCGCGGTACAGATATCATTTTATTATCGCAGGCGGCTACTAAAAAT  
CCAOCACGACAGCAGACCGCTGCAGTCAACCCCAACCGATCTCTGAGAGAAT  
AATGACGAGCGCACGCTGGCGCGGCCACCAAGTTGACCAGCAGCGCTGAATCGCGCT  
GCCGAAGTCCGGCGTGTCAATTATCCGCAGAACAAAATGAGAACCTCGCGGAG  
CGCGTTTTCTGCGCGGACGTCGGCTTAATTTAGCGCTTGGCTATGCGCCAGCGC  
AGAACAACCATTTCTGATGCTGAGATTTGGGATATGGTTTCCCAAAAATAT  
ATCGGCGGATAGGTGACAGCTATCTCGGCGTTTATGAAAACGCTCTGCGCAGTC  
TATACCGATTTTTATCAGGCGTTTCAAGTGATATTCTTTCCAAAATGGGAGGCT  
GGTTATTACCAGGTAAGGACGGTAATACCGTTAAGCTAGATGTTACCTCAC  
TCAAAAATGATTTAAACAGTTTAGTCAATAAATATAATCAAAATAAACA  
GTAAATACCGTTTTATTTCCACGCGCAGTCAGGCAGCGCGCTTAAAGTAGCCAC  
TGAAGCGGAAGCGAGACAGTGGCTCAGTGAATTGAATTTACCGAATAGCTG  
CTTGAAATCTTAATGGATCCGGTTATGTCGTCACCGTTGATCTGACGCCATTAC  
AAAAAATGGTTCAGGATAATTGATGGTTTAGGCGCGCCGGGAAAAGACTCA  
AAACTTCGAAATGGATAACGCCAAATATCAAGCTGCCAGTCCGGTTTTAA  
AGCGCAGGAAGAAAATATGAAAACCACATTACAGACGCTGACGCCAAAAA  
TATAGCAATGCCAATTCAATGTACGACAACCTGGTAAAAGTGCTGAGCAGT  
ACGATAAGTAGCAGCCTCGIAAACCGCCAAAACGCTTCTGCAAGGATAACA  
GAAAGAGATATTAATAATGCTTACAAGTGTAAGGACTCAGCCCCCGTCA  
TAATGCCAGGTATGCAGACCGAGATCAAAACGCGAGGCCACGAATCTTCKGG  
CGAATCTTCKKTCAGTCAGAGAAAGTGCCACACCGACCGCTGTACGGGAAA  
TTAAAGGCCCGCAACTGGAAGATTTTCKKCGCGCTGATCAAAACAGCGAGTCT  
CGATGC

GAGCTCAGCAAC JTOGAAAGCCTGTAAAATCATG JGTCTCGCGCGATA  
CGTTTTACCGTTATCGTGAACCTGGCCGATGAAGGCGGCGTTGATGCGCTGAT  
AAATCGTAGTCGCCCGCTACCTAACCTTAAGAAACCGTAACCGATGAGGCAAC  
TGAGCAAGCTGTTGTTGATTATGCCGTTGCGTTCCCGGCCCATGGTCAGCAAC  
GAACTGCCGAAACAGGACGTTTTTATCTCCGCTAGTGATGTCCATTCCGTCI  
CGCTGCCGACAAACCTTGAGAACTTCAAAAAACGCCTGAAAGCGCTGGAAGA  
AAAAGTGGCCCGCGATGGCAITGAACTGACTGCCAGATCCCCCGCTGGAGC  
GTAAAGCCAGTGATGATGAAGCCTGTGGTGAGATTGAAACCGTTATCCGG  
GATATCTGGGGTCACAGGACACGTTCTACGTGGGCAACCTGAAAGGCGTTGG  
GCCAATCTATCAGCAGACGTTCTGTTGATACATACTCGAAGGTGGCTCACTGC  
AAGCGCTATATCACCAAAACGCCGATTACAGCGGCTGATTTGCTGAATGAT  
CGTGTACTGCCGTTTATGAGTCTCAGGGCCTGCCGATGCTAAGGATACTGAC  
AGACACGGGTACAGAATATTCGGGCAAAAGTGGAAACATCATGATTATCAGC  
TTTATCTGGAGATAAATGACATCGAACACACGAAAACGAAGGCGATGTCC  
CCGCAGACCAATGGCATCTGCCGAGCGGTTCCATAAAACGATACTGAACGAA  
TTTTATCAGGTGACGTTCCGCAAAAAGTTATATGGCGATTTTGATACATTA  
CAATCGGATCTTGATGAATGGCTGGTTCACTATAATAATGAGCGAAOCCAT  
CAGGGAAAAATGTGCTGTGKCCGGACGCCGATCGAAACGTTACITGATGGA  
AAACGCATCTGGTCTGAGAAGAAATTTAAGCCAGATGTAATCTGACAGATA  
CCTGTATAAATAACCGGTAACCTGTCAGATCAGGTCTGAGCTAATACAACT  
AATTGTATGTTATTTGTGCTTTATTGCTAAATATATATCGTTAATTGAAG  
GCITGATGCGTGTGTCTGCGTTAATCTCTTTTCATTGTGCTGTAAATTAGGC  
AGTGGAAATATGTTAATATCCGCAATACACAACCTTCTGTAAGTATGCAG  
GCTATTCCTGGTGACGCGGCACAGAGGCATCTCCGGAAGAAATTGTATGGG  
AAAAATTGAGGTTTTTTTTCCCGCAGGAAAATTACGAAGAAGCGCAACAGT  
GTCTCGCTGAACCTTTGCCATCCGGCCCCGGGAATGTTGCCTGATCATATCAGC  
AGCCAGTTTGCGCGTTTAAAAGCGCTTACCTTCCCGCGCTGGGAGGAGAATA  
TTCAGTGTAAACAGGGATGGTATAAATCAGTTTTGTATTCTGGATGCAGGCA  
GCAAGGAGATATTGTCAATCACTCTTGATGATGCCGGGAACTATACCGTGA  
ATTGTACAGGGGTACAGTGAAGCACATGACITCATCATGGACACAGAACCGG  
GAGAGGAATGCACAGAATTCCCGGAGGGGGCATCCGGGACATCCCTCCGCCCT  
GCCACAACGGTTTCACAGAAGCCAGCAGAGTATGATGCTGTCTGTCAAAT  
GGGAAAGGGATGCCACCAGGAGAGTCACCCGGCCCGGCAGCAGTGGTACA  
GGAAATGCGTGATTGCCCTGAATAACGGCAATCCAGTGCTTAACGTG  
GGAGCGTCAGGTCTTACCACCTTACCAGACCGTTTACCACCGCATAT  
TACAACACTGGTTATTCCTGATAATAATCTGACCAGCCTGCCGGAG  
TTCGCGGAAGGACTACGGGAGCTGGAGGTCTCTGGTAACCTACAAC  
TGACCAGCCTGCCATCGCTGCCGCAGGGACTACAGAAGCTGTGGGCC  
TATAATAATTGGCTGGCCAGCCTGCCGACGTTGCCGCCAGGACTAGG  
GGATCTGGCGGTCTCTAATAACAGCTGACCAGCCTGCCGGAGATGC  
CGCCAGCACTACGGGAGCTGAGGGTCTCTGGTAACAACCTGACCAGC  
TGCGCGCGCTGCCGTGAGGACTACAGAAGCTGTGGGCCCTATAATAA  
TCGGCTGACCAGCCTGCCGGAGATGTCCGCAGGACTACAGGAGCTGG  
ATGCTCTCTATAACAGCTGACCCGCCTGCCGCAAAGCCTCACGGGT  
CTGTCTTCAGCGGCACGCGTATATCTGGACGGGAATCCACTGTCTGT

ACGCACTCGTGACAGGCTCTGCGGACATCATTGGCCATTCAAGGCATC  
AGGATACACTTCGATATGGCGGGGGCTTCCGTCCCCGGGAAGCCCCG  
GCACTGCACCTTGGCGGTGCTGACTGGCTGACGTCTGCACGGGAGGGG  
GAAGCGGCCCCAGGCAGACAGATGGCAGGCGTTCGGACTGGAAAGATA  
ACGCCGCGCGCTTCAGCCTGGTCTTGGACAGACTGCGTGAGACGGAA  
AACTTCAAAAAAGACGCGGGCTTTAAGGCACAGATATCATCCTGG  
CTGACACAACCTGGCTGAAGATGCTGCGCTGAGAGCAAAAACCT  
TTGCCATGGCAACAGAGGCAACATCAACCTGCGAGGACCGGGTGAC  
ACATGCCCTGCACCAGATGAATAACGTACAACCTGGTACATAATGC  
AGAAAAAGGGGAATACGACAAACAATCTCCAGGGGGCTGGTTTCCAC  
GGGGCGTGAGATGTTCCGCCCTGGCAACACTGGAACAGATTGCCCGGG  
AAAAAGCCGGAAACACTGGCTTTAGTTCGATGACGTTCAGGTCTATCT  
GGCGTTCCAGAATAAGCTGAAGGAATCACTTCAGCTGACCAGCGTG  
ACGTCAGAAATGCGTTTCTTTGACGTTTCCGGCGTGACGGTTTCA  
GACCTTCAGGCTGCGGACGTTCAGGTGAAAAACCGCTGAAAACAGCG  
GGTTCAGTAAATGGATACTGCAGTGGGGGGCCGTTACACAGCGTGCT  
GGAACGCCAAAGTGCCGGAAACGCTTTAACGCGCTTCGTGAAAAGCA  
AATATCGGATTATGAAGACACGTACCGGAAGCTGTATGACGAAGT  
GCTGAAATCGTCCCCGGCTGGTTCGACGATACCGATGCAGAACGTACT  
ATCGGAGTAAGTGCGATGGATAGTGCGAAAAAAGAATTTCTGGA  
TGGCCTGCGCGCTCTTGTGGATGAGGTGCTGGGTAGCTATCTGACAG  
CCCCGTGGCGTCTTAACTGAGCACGATATTCTCCGCACCAGGCCAATGTG  
GTGCGGTGAACAAAGATATTCTTGGACAAAACAACATGACACAGCACTGA  
TGATGCACAGGTGAAACAGGGGAGACITCTTCACTCAGGGCGTACGCCAACTC  
AACCTTTTCCAGGATACGCC/

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/18504

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 536/22.1, 23.7; 435/7.2, 7.32, 7.35, 29, 30, 32, 38, 252.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22.1, 23.7; 435/7.2, 7.32, 7.35, 29, 30, 32, 38, 252.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog

search terms, Salmonell7, secreted proteins, Ssp7, prg7

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUECK et al. <i>Salmonella typhimurium</i> secreted invasion determinants are homologous to <i>Shigella</i> lpa proteins. Molecular Microbiology. 1995, Vol. 18, No. 3, pages 479-490, see entire document.	1, 2, 4, 10, 16
X	HANTMAN et al. Defective Extracellular Protein Secretion by <i>Salmonella typhimurium</i> Mutants Impaired in Induction of Eucaryotic Cell Membrane Ruffling and Macrophinocytosis. Abstracts of the 95th General Meeting of the American Society of Microbiology. 21-25 May 1995, page 184, Abstract B-109, see entire abstract.	17-19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

12 FEBRUARY 1997

Date of mailing of the international search report

21 MAR 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

H. F. SIDBERRY

Telephone No. (703) 308-0106

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/18504

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

[illegible]

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/18504

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 19/00, 21/00, 21/04; C12N 1/21; C12Q 1/02, 1/10, 1/18, 1/24; G01N 33/569

## BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

A sequence listing is required for all disclosures of sequence information in which the sequence has four or more amino acids or ten or more nucleotides. The PCT application does not comply with Section 1.821(e), (a printable copy of the "Sequence Listing") a computer readable form, as defined in Sections 1.82(c), 1.822 and 1.823, recorded as a single file on either a diskette or a magnetic tape. The claims recite Sequence Identifiers, but do not comply with the stated requirements, and therefore, no meaningful search could be carried out on claims 3,5, and 11.

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1, 2, 4, 6-10, and 12-19, drawn to DNA encoding a Salmonella secreted proteins (Ssp), cell, method of inducing the uptake of a bacterial cell.

Group II, claims 20 and 22, drawn to SspC polypeptide.

Group III, claims 23 and 25, drawn to SspD polypeptide.

Group IV, claims 26 and 28, drawn to SspH polypeptide.

Group V, claims 29 and 31, drawn to lagB polypeptide.

Group VI, claim 32, drawn to antibody which binds a Ssp.

Group VII, claim 33, drawn to methods of detecting a Salmonella infection using DNA.

Group VIII, claim 34, drawn to method of detecting a Salmonella infection using antibody.

Group IX, claims 35 and 36 drawn to method of targeting an antigen to an epithelial cell.

Group X, claim 37, drawn to method of inducing a cytotoxic T cell response.

Group XI, claims 38-41, drawn to vaccines comprising attenuated bacteria and a method of vaccinating.

Group XII, claim 42, drawn to transformed Salmonella.

Group XIII, claim 43, drawn to a method of vaccinating.

Group XIV, claim 44, drawn to StpA polypeptide.

Group XV, claim 45, drawn to method of dephosphorylating a protein.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

The claims are deemed to correspond to the species listed above in the following manner:

species of DNA

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/18504

DNA(SspA), claims 8-10; SspB, claims 2, 10; SspC, claims 4, 10; SspD, claims 6, 7, 10; SspH, claims 12 and StpA, claims 14.

The following claims are generic:

For the DNA, claims 1, 10, 16-19.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: because each DNA encodes a protein which differs physicochemically, antigenically, structurally and functionally.